EXPERIMENTAL CELL RESEARCH

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VOLUME 15 · AUGUST 1958 · NUMBER 1

ACADEMIC PRESS INC., PUBLISHERS
NEW YORK and LONDON

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Experimental Cell Research **15**, *1–20* (*1958*)

HISTOCHEMICALLY DEMONSTRABLE PHOSPHATASES AND PROTEIN SYNTHESIS

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Received October 1, 1957

The distribution and activity of histochemically demonstrable phosphatases in protein-synthesizing cells has not yet been sufficiently investigated. Fell and Danielli [16] earlier noticed the high activity of alkaline phosphatase in fibroblasts and in the newly formed collagen fibres. Bourne [4] made similar observations in the healing of bone lesions. In the first stage of the healing protein fibres rich in phosphatases were formed.

Also Bradfield [7], investigating the silk glands of insect larvae, found a high alkaline phosphatase activity in the apical parts of the secreting cells of the glands. In his opinion, the alkaline phosphatase either liberates the produced protein from the nucleoproteid complex, or constitutes an integral part of the newly formed proteins. Lesher [26], observing the salivary glands in *Drosophila*, suggests that the high activity of the alkaline phosphatase in the apical parts of the cells may be connected with the transport of secretion to the lumen of the gland. In the opinion of Denucé [14] and Robertson *et al.* [31] the activity of the alkaline phosphatase is not connected with the synthesis of proteins, but exclusively with the transport of the secretion across the cellular membrane.

Dempsey *et al.* [13] attribute to alkaline phosphatase an important role in the transport of nutritional substances across the membrane. The high activity of the enzyme in the capillary vessels may be interpreted as evidence of this action. Because after thyroid gland stimulation the activity of the alkaline phosphatase is diminished, while the activity of the acid phosphatase is distinctly increased, participation of the latter in the synthesis of the secretion is postulated by the above mentioned authors [13].

These various and often opposed conclusions of the histochemists are evidence that the distribution and activity of the phosphatases during the synthesis of the proteins requires a more detailed analysis. This problem seems especially interesting in the light of the last investigations of Ellis *et al.* [15] who, by quantitative chemical methods, have found that in the reticulocytes of the rabbit the activity of certain phosphatases is considerably higher than in the erythrocytes, which are homological cells not synthesizing proteins.

For that purpose we decided to study, on a sufficiently large amount of experimental material, the activity and the distribution of certain phosphatases by the use of histochemical methods and to try to answer the following questions:

(1) What is the distribution and the intensity of the histochemical reaction of alkaline phosphatase, acid phosphatase, 5-nucleotidase and adenosinetriphosphatase[1] in standard tissue systems which are characterised by a rapid protein synthesis?

(2) Which of the investigated enzymes are associated with the protein synthesis and, at the same time, which cellular structures take part in this process?

The present work is an attempt to associate the results of the quantitative findings of other authors with the morphological pictures obtained with the use of histochemical methods.

MATERIAL AND METHODS

The material investigated was divided into six experimental groups, composed of tissue systems characterised by an extensive protein synthesis.

Experimental group I.—In this group the method described by Borsook [2], Holloway [19], and applied in the experiments of Ellis *et al.* [15] was used. Borsook [2] found that the reticulocytes, in contrast with erythrocytes, are characterized by a rapid protein synthesis. The difference in enzyme activity in the two types of cells, therefore, can, according to Borsook's opinion, be connected with the ability or lack of ability to synthesize.

For the experiment 7 male chinchilla rabbits aged 6–9 months were used. Reticulocytosis was produced by subcutaneous injection of 1 cc of a 2.5 per cent solution of phenyl-hydrazine hydrochloride in Ringer's solution daily for a period of seven days. Twenty-four hours after the last injection blood was collected from the marginal vein of the ear and smears were made on slides. Smears of normal blood were collected from the same animals prior to the injections. Because we are convinced that fixation in acetone causes a decrease of the activity of ATP-ase, the smears were dried for about 10 minutes in exsiccators over P_2O_5 and then incubated for a period of 5 hours. The composition of the incubating mediums and the conditions for the detection of the phosphatases are given at the end of this section.

Experimental group II.—According to Caspersson [8], the pancreatic cells are able to produce during one day a quantity of protein secretion equal to their own mass, and they are therefore an adequate material for the investigation of the protein synthesis process. In this group the method of Caspersson *et al.* [9] was used. Thirteen 6-months-old male white mice from our stock colony were divided into three subgroups:

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[1] In the present paper the term adenosine triphosphatase is used to include all enzymic activities implying the liberation of inorganic phosphate from ATP (cf. Discussion, p. 16).

(1) Four control mice, which, for the purpose of reducing to a minimum the production of secretion, were starved for 24 hours before sacrificing them.

(2) Four mice which received one 1 cc subcutaneous injection of 1 per cent pilocarpine hydrochloride 25 minutes before they were killed, for the stimulation of the excretory processes.

(3) Five mice each of which received 0.3 cc of 1 per cent pilocarpine hydrochloride and were slain after a lapse of 3 hours (2 mice) and 4 hours (the remaining 3 mice). In this 3–4 hour period there is an intensive regeneration of the secretory substances previously removed by pilocarpine.

All mice were killed by decapitation. The pancreas was immediately removed and frozen at the temperature of dry ice (ca. −70°C).

Experimental group III.—According to the investigation results of Stowell [34], Lee *et al.* [25] and Hultin,[1] after a partial hepatectomy in the rat, there is a strong stimulation of the protein and nucleic acids synthetic processes in the remaining, regenerating part of the liver.

The experiment was carried out on 10 9-months-old female rats of the Wistar strain. Under ether anesthesia, the left lateral and median lobes of the liver were removed. Two animals were slain by decapitation 24 hours after the operation, 5 others after 48 hours. The liver lobes removed during the operation were utilised as control material. Before and after the operation the animals were fed on a standard diet and received water ad libitum.

Because during the investigations the necessity arose of examining a regenerating liver in which the synthesis of deoxyribonucleic acid was inhibited, hepatectomy was performed on three other rats. According to the directions given by Potter *et al.* [30], these animals were irradiated with 2000 r on the whole body (180 kV, 15 mA, 2 mm Al, 142,6 r/min.). Directly after the irradiation, the hepatectomy was performed under ether anaesthesia. The rats were slain by decapitation 24 hours after the operation.

Experimental group IV.—The ascites tumors of mice are characterised by a rapid growth, and owing to that they make adequate material for the investigation of the protein synthesis and of the nucleic acids. In our experiments we used the Ehrlich ascites carcinoma and the amytal ascites sarcoma developed in mice by Juhasz *et al.* [23] with amytal-sodium. The material for the examination was grown for 7 days in male mice from our stock colony, and then collected by peritoneal puncture. The smears on the slides were dried in exsiccators, just like the blood smears, and incubated for 5 hours in suitable media. Because in the course of the investigations the necessity arose of inhibiting the synthesis of DNA in the cells of the Ehrlich ascites carcinoma, 7 mice were submitted to irradiation in conformity with Forssberg and Klein's method: 1700 r applied on the abdomen, 180 kV, 15 mA, 2 mm Al, distance 40 cm. The material was collected 1, 3, 6 and 24 hours after irradiation.

Experimental group V.—For the investigation of the activity and distribution of phosphatases during the synthesis of collagen, the experimental material of K.

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[1] Hultin T. and von Decken, A., *Exptl. Cell Research* **13**, 83 (1957).

Vorbrodt [37] was utilised, which produced granuloma pouches in rats. The experiments were carried out on rats of the Wistar strain, male 6–8 months old. The granuloma pouches were produced by a modified method of Sely [32]. After depilation of the dorsal skin of the rats, 22 cc of air was introduced subcutaneously, followed by 0.5 cc of a 0.5 per cent solution of croton oil in olive oil. Five days after this operation 0.5 cc of 3 per cent croton oil was injected, and after 10 days 0.5 cc of pure croton oil. The material was collected after 1, 3, 6, 17, 23 and 38 days of the development of the pouch. The walls of 10 such granuloma pouches were examined.

Experimental group VI.—In this group the silk glands of wax moth larvae (*Galleria mallonella* L.) were examined. In the opinion of Allegret [1], the secretion is produced in the cytoplasm of the glandular cells, with no appearance of definite constituents of cytoplasm; in the apical part of the gland the fibrous protein, the so-called fibroin, is formed. In our investigations we used larvae which were in the IIIrd, IVth and, mainly, the VIth stage of development. The larvae were frozen in dry ice and then sectioned longitudinally in the cryostat.

All the tissues, with the exception of the blood smears and the ascites tumors, were frozen at the temperature of dry ice (ca. -70°C) and cut in the Linderström-Lang cryostat into sections 10 μ thick. After a short drying in the exsiccator over P_2O_5, the sections were incubated for 150 minutes at a temperature of 37°C in adequate incubating media. The smears of blood and of the ascites tumors were incubated for 5 hours. The control sections were incubated in the same way after previous inactivation of the enzymes in 1 N HCl for 30 minutes.

The alkaline phosphatase (AlPh) was detected according to Gomori's method [17], with an 0.06 M concentration of $CaCl_2$ in the incubating medium, and at pH 9.4.

5-Nucleotidase (5-N) was determined by Gomori's method [17], also with the concentration of the $CaCl_2$ lowered from original conc. to 0.06 M, pH 8.5.

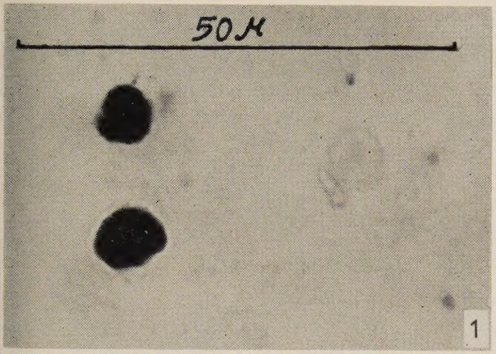
Acid phosphatase (AcPh) was detected by Gomori's method [17] at pH 5.0.

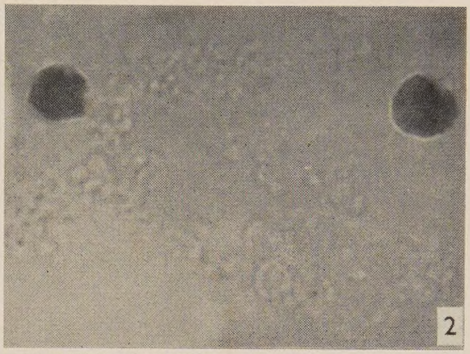
Adenosine triphosphatase (ATP-ase) was detected by a slightly modified method of Naidoo and Pratt [27], and where $MgCl_2$ was used exlusively as activator in a concentration of 0.005 M, pH of the solution 6.5. The detailed composition of the incubating media has been given in our earlier work [36]. The localisation of the activity of AlPh and 5-N was shown by the CoS method, and the localisation of the activity of AcPh and ATP-ase by the PbS method. For the histological examination, the mounted sections were fixed for 30 minutes in Carnoy's fluid and stained with haematoxylin and eosin or with 1 per cent solution toluidine blue at a pH of 4.5.

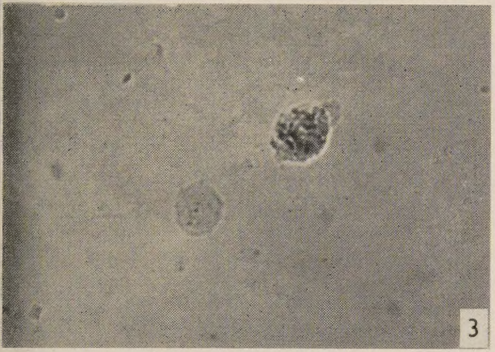
RESULTS

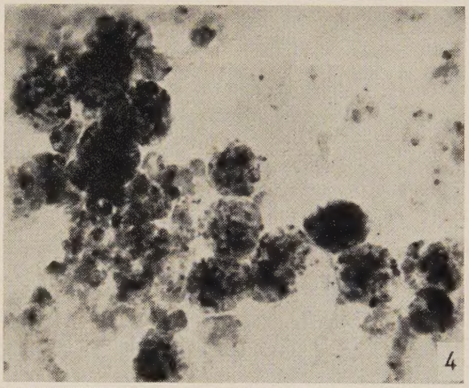
First group: reticulocytes and erythrocytes of rabbit.—The reaction for AlPh in both the erythrocytes (Fig. 1) and the reticulocytes (Fig. 2) is completely negative. Only the leucocytes contain the enzyme, as is shown in the attached microphotographs. Because the reactions to 5-N are identical, we are not inserting the corresponding microphotographs.

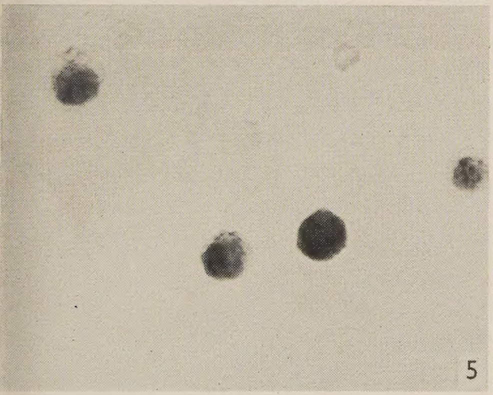
In the normal blood AcPh is to be found exclusively in the white blood











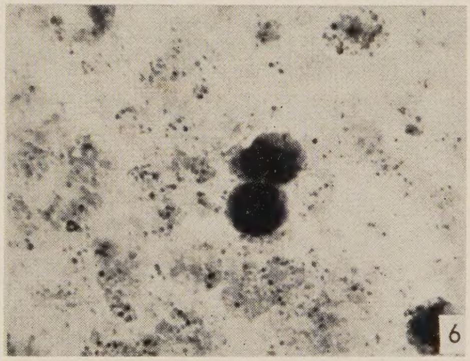


Fig. 1.—Lack of positive reaction to alkaline phosphatase in the erythrocytes, strong reaction in two leucocytes.

Fig. 2.—Absence of reaction to AlPh in reticulocytes, positive reaction in two leucocytes.

Fig. 3.—Absence of reaction to acid phosphatase (AcPh) in erythrocytes, weak, granular reaction in leucocytes.

Fig. 4.—Strong positive reaction to AcPh in cytoplasmic granules of the reticulocytes.

Fig. 5.—Absence of reaction to ATP-ase in erythrocytes, positive reaction in four leucocytes.

Fig. 6.—Positive reaction for ATP-ase in minute cytoplasmic granules in the reticulocytes. Three leucocytes give a strong reaction in the whole cell.

cells. The erythrocytes do not reveal the presence of the enzyme (Fig. 3). In the reticulocytes, the reaction to AcPh is usually strong and is localized mainly in the cytoplasmic granules (Fig. 4). The white blood cells, which appear in a considerable quantity among the reticulocytes, also reveal AcPh activity in their cytoplasm.

In the smears of normal blood the ATP-ase appears exclusively in the white blood cells, which is no sign of activity in the erythrocytes (Fig. 5). By contrast, in the reticulocytes the activity of this enzyme is evident and is distributed in the small cytoplasmic granules (Fig. 6).

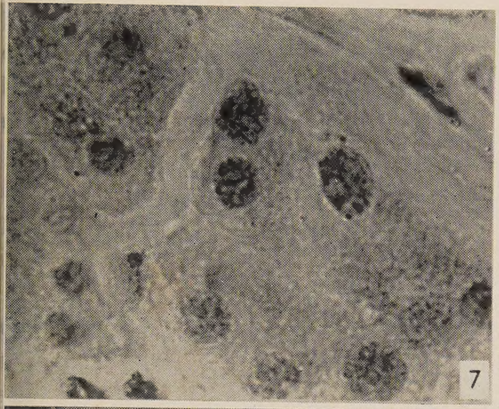
Second group: the pancreases of mice.—In the glandular cells of the pancreas we did not observe any activity of AlPh or of 5-N. These enzymes are present only in the walls of the small blood vessels. Neither did we observe comprehensible differences in the reaction intensity and in the distribution of the examined phosphatases between the pancreas of the control animals and experimental ones. For that reason we do not insert microphotographs illustrating the distribution of these enzymes.

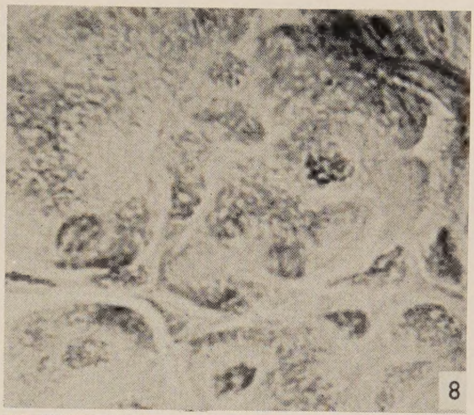
The reaction to AcPh in the pancreas of control animals is localised mainly in the cytoplasm and in the nuclei (Fig. 7). This nuclear reaction in many cells gives the impression that the enzyme is not to be found in the inside of the nucleus but on its surface.

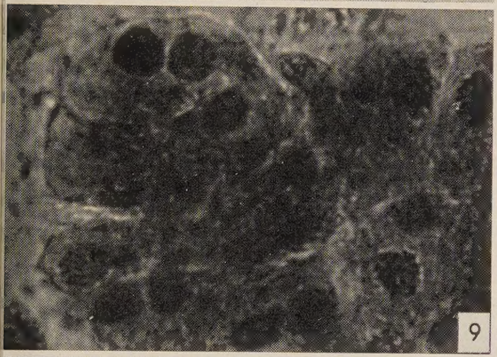
At 25 minutes after the injection of pilocarpine, the activity of the enzyme disappears around the nuclei and in the basal part of many cells (Fig. 8), while in the apical parts of the cells, the activity of AcPh is visible in the middle of the granular secretion. The nuclear reaction is well differentiated and generally has the characteristics of granular-fibrous structures adhering to the nuclear membrane. Three and four hours after the injection of pilocarpine, the activity of AcPh in the cytoplasm is distinctly increased. The enzyme is localised in the granules, which are distributed equally in the basal as well as the apical parts of the cytoplasm (Fig. 9). The nuclear reaction is also increased, though to a lesser degree.

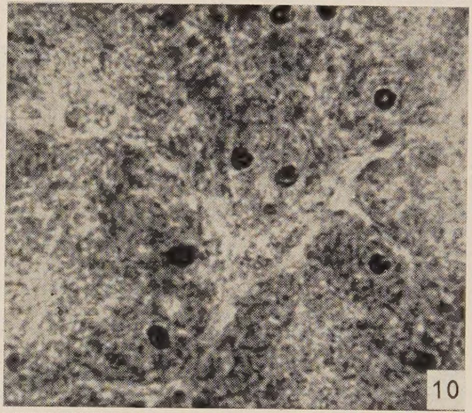
In the pancreas of the control animals, the activity of ATP-ase is localised mainly in the cytoplasmic granules grouped in the basal parts of the cells and around the nuclei, while the nuclei themselves are completely or almost completely free of the enzyme (Fig. 10). The nucleoli are characterised by a high activity of ATP-ase. Twenty-five minutes after the injection of pilocarpine, the activity of that enzyme is distinctly weaker in the cytoplasm, while the nucleoli are smaller and contain less ATP-ase (Fig. 11) than in the control animals.

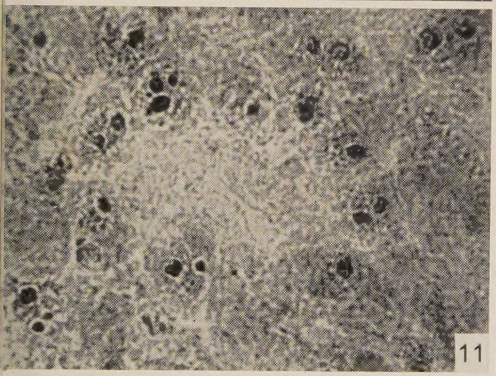
Three and four hours after the injection of pilocarpine the intensity and











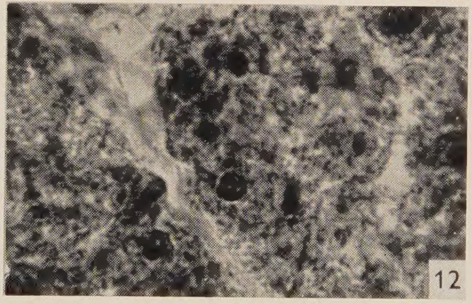


Fig. 7.—Reaction to AcPh in pancreas of mice starved for 24 hours.

Fig. 8.—Reaction to AcPh in pancreas of mice 25 minutes after pilocarpine injection.

Fig. 9.—Very strong reaction to AcPh in pancreas of mice 4 hours after the injection of pilocarpine.

Fig. 10.—Distribution of the activity of ATP-ase in pancreas of starved mice. The absence of reaction in nuclei but strong reaction in the nucleoli is significant.

Fig. 11.—Reaction to ATP-ase in pancreas of mice 25 minutes after the injection of pilocarpine.

Fig. 12.—Reaction to ATP-ase in pancreas of mice 4 hours after the injection of pilocarpine. The nucleoli are distinctly enlarged and rich in enzyme.

distribution of the reaction of ATP-ase is generally similar to that observed in control pancreases, and the nuceloli rich in enzyme are evidently enlarged (Fig. 12). The reaction in the nuclei continues to be weak or negative.

Third group: the regenerating liver of rat.—The reaction to AlPh in the normal liver cells is, strictly speaking, negative. The enzyme is present only in the walls of the blood vessels and in single migrating cells (Fig. 13). In the regenerating liver we do not observe changes in the intensity and distribution of that enzyme (Fig. 14).

The activity of 5-N is localised mainly in the walls of the hepatic ducts, on the border of the cells, in the cytoplasmic granules and in the nuclei (Fig. 15). In the regenerating liver the cytoplasmic granules containing this enzyme as well as the nuclei are slightly larger (Fig. 16). Apart from that we do not note any evident differences in the intensity of the reaction to 5-N, between the normal and regenerating liver cells.

The activity of AcPh in the normal liver cells is localised mainly in the cytoplasmic granules grouped in the region of the nucleus and in other cytoplasmic structures, appearing as blurred regions (probably clumps of microsomes). Some of the nuclei give a very weak, hardly visible positive reaction (Fig. 17). Twenty-four and 48 hours after hepatectomy the activity of AcPh is markedly increased mainly in the mentioned cytoplasmic structures, which seem to be basophilic substances formed probably by the microsomes (Fig. 18). Some of the nucleoli also reveal the presence of the examined enzyme.

In the normal liver cells the reaction of ATP-ase is distinct enough in the granules disseminated throughout the cytoplasm. The nuclei and especially the nucleoli contain more enzyme than the cytoplasm (Fig. 19). Twenty-

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Fig. 13.—Reaction to AlPh in normal liver. Absence of enzyme in liver cells, strong reaction in the wandering histiocyte.

Fig. 14.—Reaction to AlPh in cells of regenerating liver (48 h. after hepatectomy); strong reaction in the histiocyte.

Fig. 15.—Weak reaction to 5-nucleotidase (5-N) in the liver cells.

Fig. 16.—Slightly higher activity of 5-N in the cells of regenerating liver.

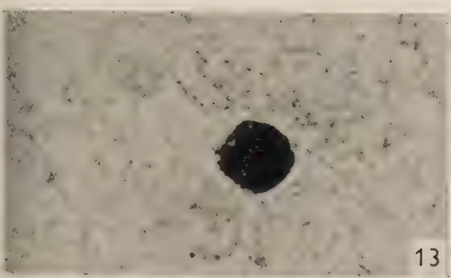
Fig. 17.—Reaction to AcPh in normal cells of the rat liver.

Fig. 18.—Markedly increased reaction to AcPh in the cells of the regenerating liver (48 h. after hepatectomy).

Fig. 19.—Reaction to ATP-ase in the normal cells of rat liver.

Fig. 20.—Strongly increased reaction to ATP-ase in the nuclei of the cells of regenerating liver.

Fig. 21.—Reaction to ATP-ase in the cells of regenerating liver after irradiation with a dose of 2000 r. Evident decrease of activity of ATP-ase in nuclei.



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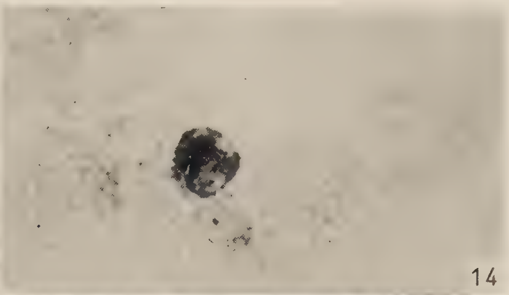

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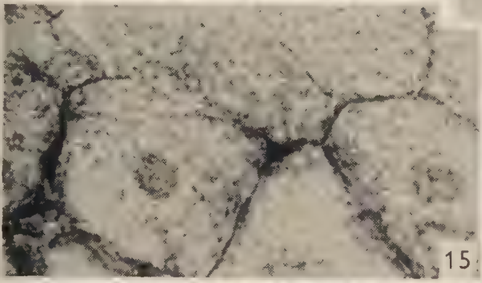

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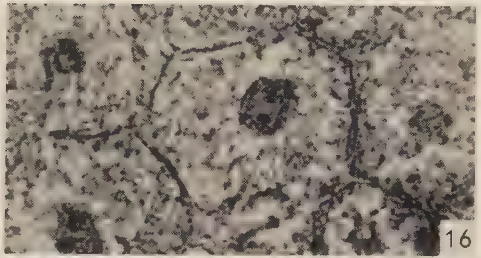

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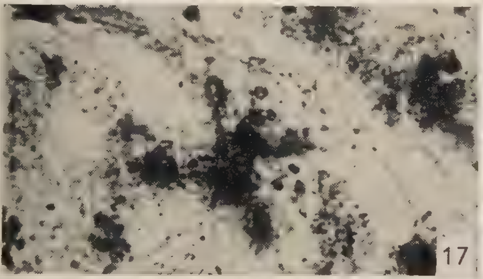

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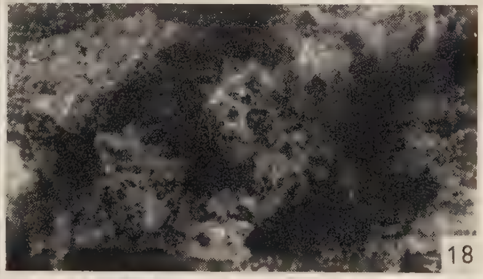

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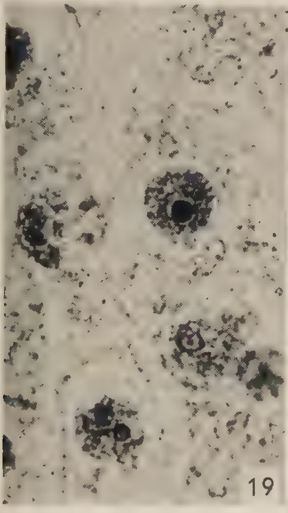

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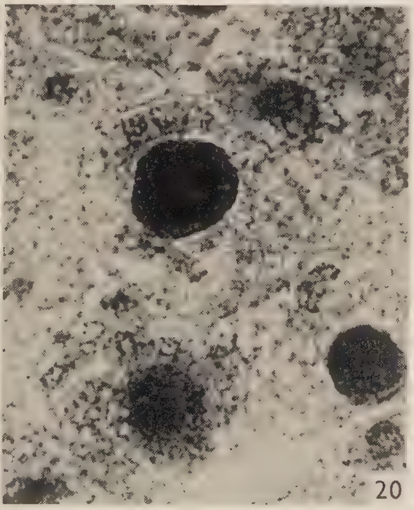

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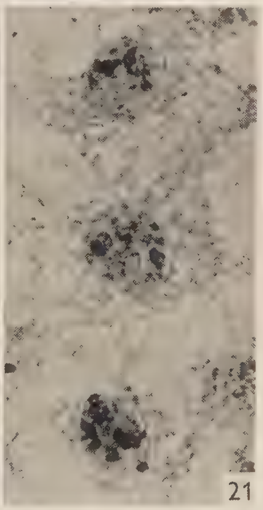

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four and 48 hours after hepatectomy the reaction to ATP-ase is distinctly increased in the nucleoli and the nuclei, while in the cytoplasm it remains unchanged or is insignificantly increased (Fig. 20).

Twenty-four hours after hepatectomy in irradiated rats with a dose of 2000 r, the activity of ATP-ase in the nuclei is distinctly weakened in comparison with the regenerating liver of non-irradiated animals. Also the reaction in the cytoplasm is distinctly decreased (Fig. 21). Proportionally, the strongest reaction is in the nucleoli of these cells.

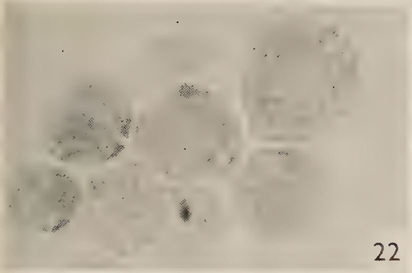
Fourth group: ascites tumors of mice.—In the cells of Ehrlich cancer (Fig. 23), as in the cells of the sarcoma (Fig. 22), we do not observe any reaction of alkaline phosphatase.

Similarly the reaction of 5-N appears only in few cells of the sarcoma, and in them is localised mainly in the cytoplasm granules and sometimes also in the nuclei or nucleoli (Fig. 24). In the Ehrlich ascites carcinoma the reaction is still weaker and appears only in certain cells. In the majority of the cells we do not observe the presence of the enzyme (Fig. 25).

The reaction of acid phosphatase is distinctly stronger and is localised exclusively in cytoplasmic granules of various size. Generally the reaction is stronger in the cells of the sarcoma (Fig. 26) than in the carcinoma (Fig. 27).

In sarcoma the ATP-ase is located in the granules of cytoplasm as well as in the nuclei and nucleoli (Fig. 28); some of the cells have a stronger reaction and some have weaker. In the cells of the Ehrlich ascites carcinoma the reaction is more uniform. A significant characteristic of these cells is the very high activity of ATP-ase in the nuclei and nucleoli (Fig. 29), contrasted with a low activity in the cytoplasm. After the irradiation of mice with a dose of 1700 r, the reaction to ATP-ase in the cells of Ehrlich carcinoma does not undergo any change for one hour. After 3 hours the activity of the enzyme is distinctly decreased in the nuclei, although it is preserved in the large, lobular nucleoli (Fig. 30). Similar pictures are observed also after 6 hours.

Fifth group: the production of collagen.—On the first and the third day after the formation of the air pouch and the introduction of 0.5 cc of 0.5 per cent solution of croton oil in the loose subcutaneous connective tissue, there can be seen numerous migratory cells and fibroblasts, which have a positive reaction to the alkaline phosphatase test. These cells are grouped around the air pouch, forming a sort of wall or a "defence barrier" as defined by Selye [32]. Inside the cavity on the third day is found a small quantity of exudative liquid, which gives a positive diffusion reaction. On the sixth day the wall of the pouch is already well formed and it is possible to differentiate an inner layer rich in fibroblasts and an outer layer in which the pro-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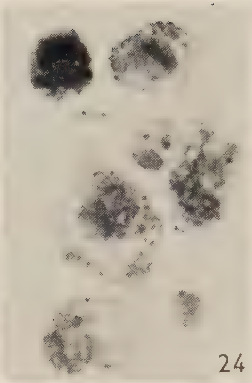
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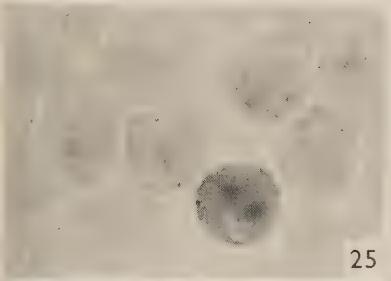
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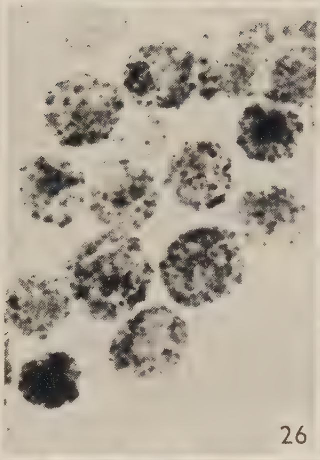
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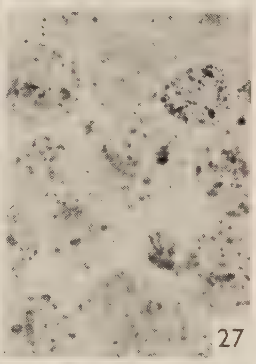
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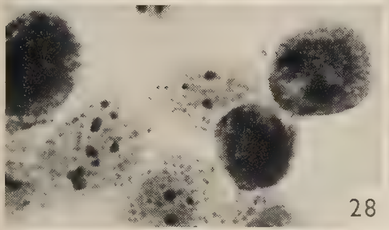
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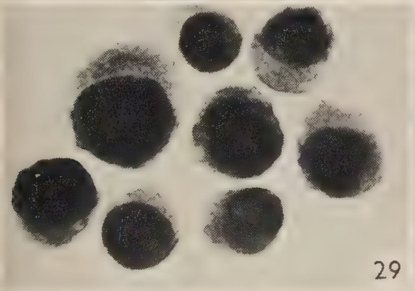
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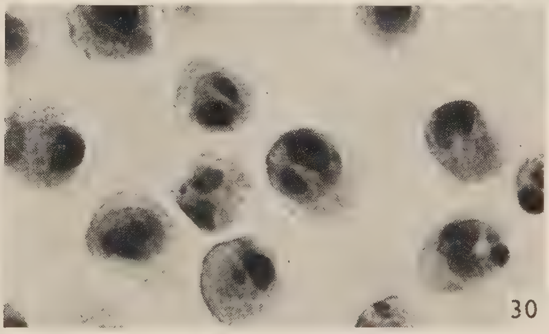
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Fig. 22.—Absence of reaction to AlPh in the cells of ascites sarcoma in mice.

Fig. 23.—Absence of reaction to AlPh in the cells of Ehrlich ascites carcinoma.

Fig. 24.—A group of cells of the ascites sarcoma giving a positive reaction to 5-N.

Fig. 25.—Very weak or negative reaction to 5-N in the cells of Ehrlich carcinoma.

Fig. 26.—Reaction to acid phosphatase in the cells of ascites sarcoma.

Fig. 27.—Reaction to AcPh in the cells of Ehrlich carcinoma.

Fig. 28.—Reaction to ATP-ase in ascites sarcoma.

Fig. 29.—Reaction to ATP-ase in the cells of Ehrlich carcinoma.

Fig. 30.—Reaction to ATP-ase in the cells of Ehrlich carcinoma in 6 hours after irradiation with a dose of 1700 r. Distinct decrease of activity of nuclear ATP-ase.

duced fibres are located. The test reaction for AlPh in the fibroblasts, both in the outer layer and in the inner one, is very weak or negative (Fig. 31), while among the fibres of the outer layer it is much stronger. Also high activity of the enzyme is observed in the walls of the newly formed blood vessels. On the 17th, 23rd and 38th day of the pouch development the distribution of the reaction is similar: weak or negative in the fibroblasts of the outer layer, but very strong in the collagen fibres which form the outer layer of the granulome wall of the pouch (Fig. 36).

The distribution of the activity of 5-nucleotidase on the first and third day is similar to the distribution of AlPh, but the highest activity of the enzyme is in the newly formed blood vessels. On the sixth day the enzyme is localised exclusively in the capillary vessels, which is obvious from the microphotograph (Fig. 33). In the later stages (17th, 23rd and 38th days) strong reaction was equally observed in the blood vessels and in the collagen fibres which form the outer layer of the pouch wall.

On the first and third day of the granular pouch development, the fibroblasts and the cells forming the inflammatory infiltration are characterised by a distinct activity of the acid phosphatase localised mainly in the cytoplasm. On the sixth day and in later stages of the pouch development, the fibroblasts gathered mainly in the inner layer give a strong reaction, indicating that the resources of the AcPh are mainly in the cytoplasm of these cells (Figs. 32 and 35). The reaction in the cells lying between the fibres in the outer layer is, as shown in the microphotographs, weaker than in the inner layer. The distribution of the AcPh activity is opposite to that of the AlPh: a strong reaction characterises the inner layers, while it is weaker in the outer layer of the pouch wall (compare Figs. 31 and 36 with Figs. 32 and 35).

Fig. 31.—Reaction to AlPh in the wall of the granuloma pouch after 6 days of development. High activity of AlPh in the collagen fibres grouped in the outer part of the pouch wall (lower left), weak activity of the enzyme in the fibroblasts forming the internal part of the wall.

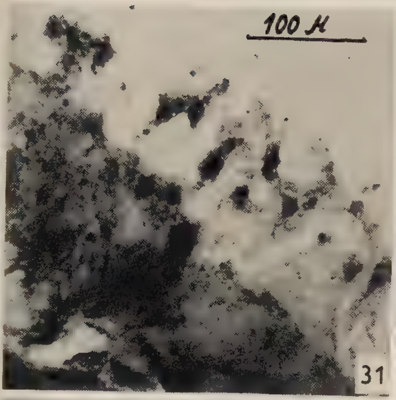
Fig. 32.—Reaction to AcPh in the wall of granuloma pouch after 6 days of growth. The distinct activity of enzyme in fibroblasts is significant.

Fig. 33.—Activity of 5-N in the wall of 6 days' pouch, localized mainly in the blood vessels.

Fig. 34.—Reaction to ATP-ase after 6 days of growth of granuloma pouch is localised mainly in the nucleoli and in the cytoplasm of the fibroblasts.

Fig. 35.—The inner part of the wall of the granuloma pouch after 17 days of growth. Strong reaction to AcPh in fibroblasts and in the newly formed fibres.

Fig. 36.—The wall of the pouch after 23 days of growth. Strong reaction to AlPh in fibroblasts grouped in the inner part of the pouch wall, while very strong reaction in the collagen fibres forming the outer part of the pouch.



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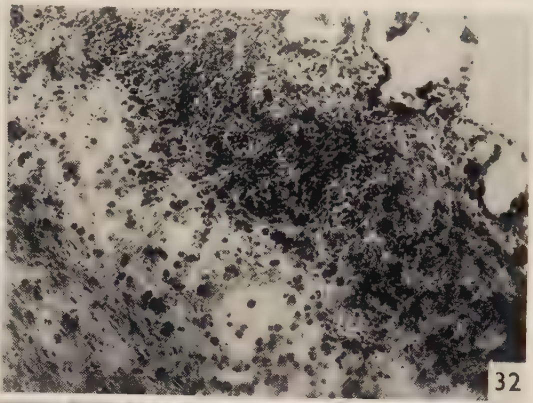

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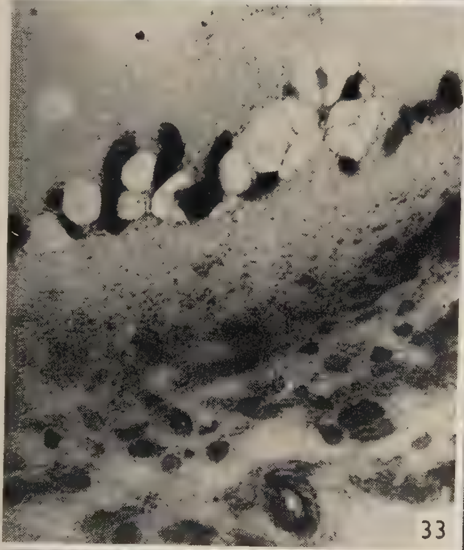

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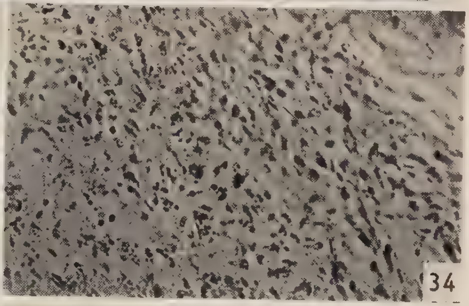

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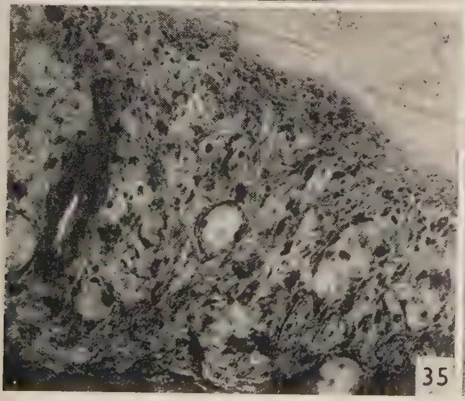

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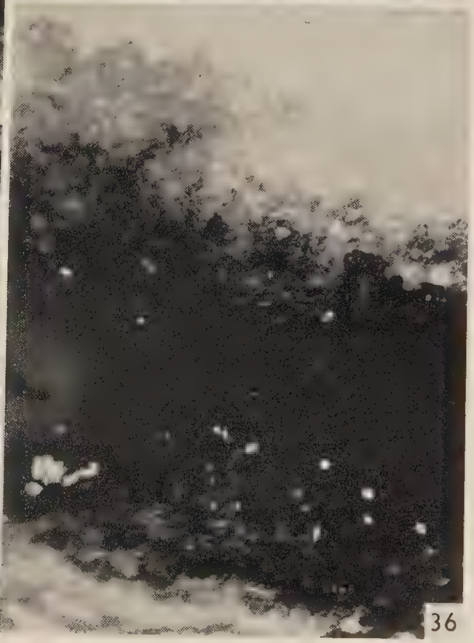

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In all stages of development of the granulome pouch, the activity of ATP-ase in the fibroblasts is localised mainly in the cytoplasm and in the nucleoli. Apart from that, the enzyme is present in the walls of the newly formed blood vessels.

Sixth group: the silk glands of the larvae of wax moth.—In the third and fourth larval stage the AlPh activity in the silk glands is very weak, and can be considered as negative (Fig. 37). During the period of greatest development of the glands, i.e. in the sixth larval stage, there is a lack of reaction also in the cytoplasm of the glandular cells. An evident activity of the enzyme however, can be observed at the border of the cells or on their external surface (Fig. 41). The activity of 5-nucleotidase is quite similarly distributed: a negative, or very weak, diffuse reaction in the cytoplasm of the glandular cells in the earlier larval stages (Fig. 38), and a positive reaction on the surface of the cells or in the marginal membrane in the sixth stage.

The activity of acid phosphatase on the other hand is very distinct in all stages with the enzyme localised in the cytoplasm and in the nuclei of the glandular cells (Fig. 39). In the sixth stage the AcPh is to be found in the characteristically arranged granules of the cytoplasm, as well as on the surface of the cells (Fig. 43).

The activity of ATP-ase in the glands of the third and fourth stage larvae is particularly strong in the nuclei and weaker in the cytoplasm (Fig. 40). In the sixth stage the ATP-ase is present equally in the nuclei and in the cytoplasmic granules distributed throughout the cell (Fig. 42).

Fig. 37.—Absence of reaction to AlPh in the silk gland of wax moth in the 4th larval stage. In the lower part of the photograph positive reacting segments of the digestive canal are visible.

Fig. 38.—Transversally sectioned glandular duct (IVth larval stage). Absence of reaction in the glandular cells to 5-N. The fragments of the digestive canal which can be seen in the left lower part of the photograph, have a strong positive reaction.

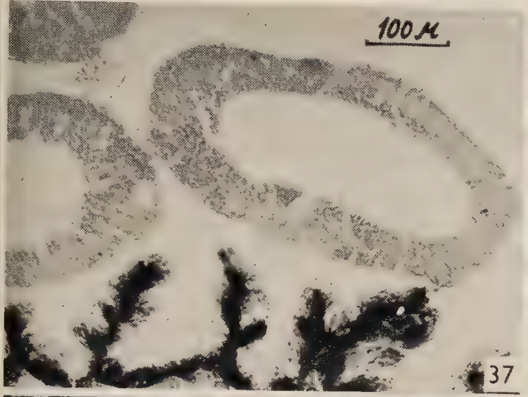
Fig. 39.—Strong positive reaction to AcPh, particularly in the basal parts of the glandular cells (IVth larval stage).

Fig. 40.—Reaction to ATP-ase in the silk gland (IVth stage). Strong activity of enzyme in polymorphus nuclei.

Fig. 41.—Oblique section of the glandular duct in the VIth larval stage. Absence of reaction in cytoplasm to AlPh, high activity of enzyme on the surface of the cells (left and right upper corner).

Fig. 42.—The fragment of obliquely sectioned glandular duct of the wax moth larvae (VIth stage). The reaction to ATP-ase is localised in the cytoplasm of the cells.

Fig. 43.—Transversally (down) and oblique (up) sectioned glandular duct in the VIth larval stage. The cytoplasmic structures are characterised by a high activity of AcPh. This enzyme is to be found also in the marginal membrane or on the surface of the cells.



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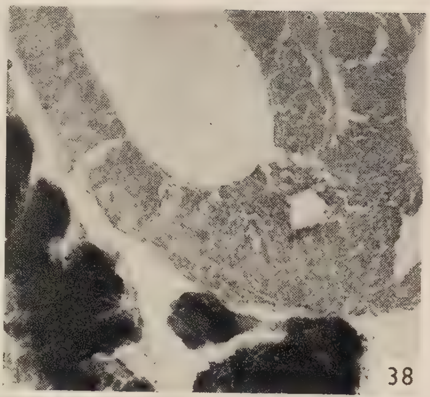

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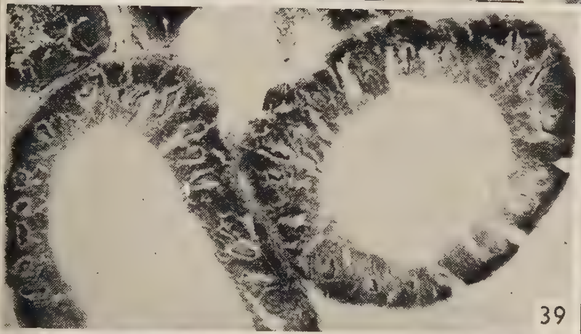

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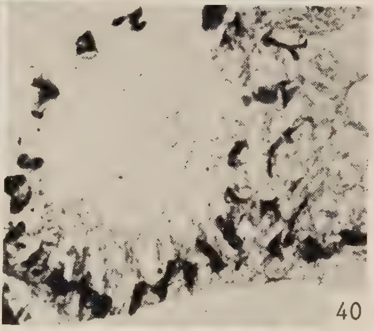

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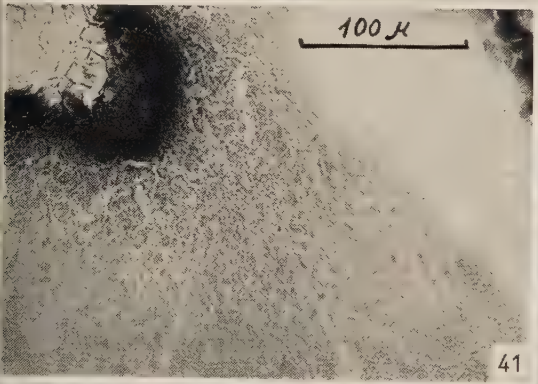

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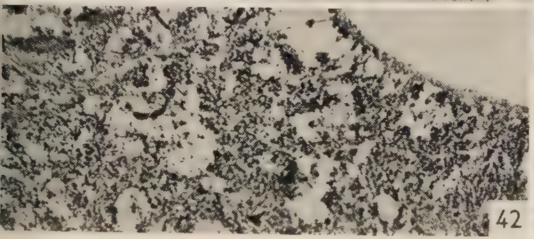

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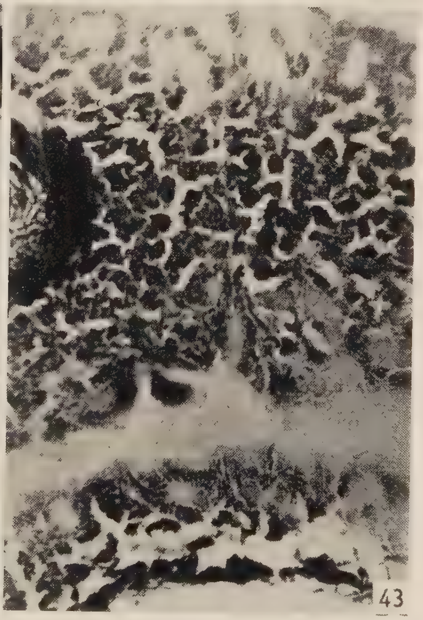

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DISCUSSION

In five of the six model tissue systems examined we observed no correlation between the AlPh activity and the process of the protein synthesis. The only exception is in the case of the fibroblasts, which showed the enzyme activity in the first days of the granular pouch formation. In the later stages, however, beginning with the sixth day, the fibroblasts forming the inner layer of the pouch wall do not contain any histochemically demonstrable AlPh, although the process of collagen production in this stage is very marked. This observation is compatible with the investigation results of Robertson *et al.* [31], who also did not observe AlPh activity during the fibrous pouch formation in the kidney region of guinea pigs.

A marked activity of the alkaline phosphatase among the already formed collagen fibres (possibly precollagen) suggests, as already expressed by Danielli [10, 11], that the enzyme in some unknown way catalyses the contraction of protein fibres at the period of collagen maturation. Unfortunately, more detailed chemical examinations are lacking. In view of the presence of the AlPh on the surface of the silk gland cells or in the cytoplasm bordering the lumen, it is suggested, in accordance with the opinion of Danielli [12], that the enzyme is engaged in the transport of the secretion from the interior of the cell to its surface. Also, our findings agree with those of Dempsey *et al.* [13], that the presence of AlPh in the walls of the minute blood vessels can be associated with its participation in the transport of the nutritive bodies from the blood to the tissues. On the basis of our observations, we can only state that we do not observe any connection between the histochemically demonstrable activity of the alkaline phosphatase and the processes of protein synthesis inside the cells. Possibly phosphatase activity is characteristic of fibrous proteins during their formation or maturation.

The complete lack of reaction to 5-nucleotidase in the reticulocytes and in the pancreas cells and a very weak activity in the Ehrlich ascites carcinoma cells and in the silk glands does not permit the association of the activity of that enzyme with the synthesis phenomenon. In the liver cells the activity of 5-N is quite marked both in the cytoplasm and in the nucleus. However, it does not undergo any definite changes during the regeneration, which is evidence of the participation of that enzyme in processes not directly associated with the protein synthesis. Only in the granular tissue was the activity of 5-N very marked, and there mainly in the fresh blood vessels and in the produced precollagen fibres. Constant appearance of 5-N in the walls of the capillary vessels could be associated with the phenomenon of active transport of various substances from blood to the cells. However, we were unable to find any papers with a detailed discussion of the role of that enzyme in the processes which interest us.

The high activity of acid phosphatase in the reticulocytes (and its lack in the erythrocytes), in tumor cells, in fibroblasts, and in silk glands, and the evident increase of its activity in the regenerating liver and in the secreting pancreas, indicates that it has some function in the protein synthetic process. Its localisation mainly in the cytoplasmic granules might imply the participation of these cytoplasmic structures in the proteosynthesis. This conforms with the results of Brachet [5, 6] obtained on the anucleated fragments of the amoebae. He found that the anucleated fragments possess the ability to produce the adaptive enzymes, which is evidence that protein synthesis occurs in the cytoplasm. In addition, the histochemical reactions indicating the presence of acid phosphatase in the cytoplasm demonstrate that our methods are valuable, because according to the results of the quantitative investigations of Palade [29] and Allard *et al.*,[1] approx. 90 per cent of the total activity of acid phosphatase in the rat liver cells occurs in the cytoplasmic fractions.

The present observations also show that ATP-ase activity is higher in protein-synthesizing cells than in the homologous cells which do not synthesize protein (e.g. reticulocytes versus erythrocytes). The presence of this enzyme in the pancreatic cells, in neoplasms, in fibroblasts and in the silk glands, permits the association of its activity with the processes conditioning the building of the protein substances. The question of the activity of ATP-ase in nuclei and nucleoli is also very interesting. In the rapidly growing neoplasmic tissue and in the regenerating liver the activity of ATP-ase is very high. Because these tissues are characterised by a rapid metabolism of desoxyribonucleic acid, we think that the activity of ATP-ase is associated here with the speed of the DNA synthesis. To demonstrate the latter we decided to inhibit the DNA synthesis with the help of adequate doses of X-ray, because, as is shown by the investigations of numerous authors [18, 24, 28, 30], one of the first effects of irradiation on tissue is the inhibition of the synthesis of that acid. The results obtained confirmed our suppositions. After irradiation, the activity of the nuclear ATP-ase was distinctly decreased with the preservation of the reaction in the nucleoli. This observation in its turn indicated that the activity of ATP-ase in the nucleoli may be associated with the synthesis of RNA or other nucleotides. This supposition was based on two facts, i.e. that the X-rays do not inhibit the synthesis of RNA [20, 35], or affect it only slightly, and the synthesis of both nucleic acids and possibly of some other nucleotides probably occurs in the nucleus [6, 21, 22].

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[1] Allard, C., Lamirande, de G. and Cantero A., *Cancer Research* **17**, 862 (1957).

It is not yet clear whether the ATP-ase in the nucleus of the cell is an integral part of the nucleoproteins (as for example it forms the integral part of myosin), or whether it is a completely independent enzyme, merely located there. Stern *et al.* [33] found that the thymonucleohistones (TNH) isolated from the thymus of rabbit hydrolyse such energy-rich compounds as ADP and ATP. The enzymatic activity of TNH is more or less comparable to the activity of the myosin ATP-ase. It seems therefore probable that ATP-ase in the nucleus is an integral component of the nucleoproteins of the desoxyribose type. This problem requires further study using biochemical methods.

Another interesting problem is the possible method of participation of acid phosphatase and ATP-ase in various stages of the protein synthesis. Borsook [3] differentiates three such stages. The first step is the activation of the carboxyl group of amino acids according to the reaction:

Amino acid + ATP $\rightleftharpoons$ amino acyl-AMP + inorganic pyrophosphate.

In the histochemical test used in this paper, it is not possible to distinguish this reaction from a true ATP-ase.

Less clear is the role of the acid phosphatase. We may presume that this enzyme is active mainly in the third step of the synthesis, when the polypeptide chain is peeled off from the polynucleotid matrix. Nevertheless this assumption should be supported by further biochemical investigations.

SUMMARY

1. With histochemical methods the activity of phosphatases was investigated in the following model tissue systems, which are characterised by an extensive protein synthesis: reticulocytes of rabbit, the pancreas of mice after pilocarpine stimulation, the regenerating liver of rat, the ascites tumors of mice, the granular tissues and the silk glands of wax moth larvae.

2. Non-fixed, frozen tissues were sectioned in the cryostat and then incubated for the purpose of detecting alkaline phosphatase, acid phosphatase, 5-nucleotidase and ATP-ase.

3. No evident association was observed between the activity of alkaline phosphatase and 5-nucleotidase and the intracellular processes of protein synthesis. The activity of these enzymes was high only in the newly formed collagen fibres, which could indicate their participation in the maturation of precollagen and collagen.

4. The high activity of acid phosphatase in all examined cells mainly local-

sed in the cytoplasm, suggests that the enzyme participates in the proteosynthesis.

5. The activity of ATP-ase in the cytoplasm is associated to a certain degree with the stimulation of the proteosynthesis. The marked activity of ATP-ase in the nuclei in the rapidly growing neoplasmic tissues and in the regenerating liver, contrasted with the low activity in the nuclei of the glandular cells (e.g. in pancreas), suggests that the enzyme participates in the metabolism of desoxyribonucleic acid. This finding was confirmed in the experiments inhibiting the synthesis of DNA with the help of high doses of X-rays.

6. The high activity of ATP-ase in nucleoli suggests that they participate in the synthesis of ribonucleic acid and, indirectly, in the synthesis of cytoplasmic proteins.

7. The results obtained in the recent investigations were compared with the bio- and cyto-chemical results of other authors.

I wish to express my sincere gratitude to Dr. Henryk Godlewski for active interest and helpful criticism. I am indebted to Dr. Lars Ernster, Wenner-Gren Institute, Stockholm, for many valuable discussions, and to Miss Molly Fudge for accurate revising of the English text.

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RECHERCHES CYTOCHIMIQUES SUR LES POLYPHOSPHATES INORGANIQUES CONTENUS DANS LES ORGANISMES VIVANTS

I. ÉTUDE DE LA RÉACTION DE MÉTACHROMASIE ET DES COLORATIONS AU VERT DE MÉTHYLE ET À LA PYRONINE EN FONCTION DE LA LONGUEUR DE CHAÎNE DES POLYPHOSPHATES

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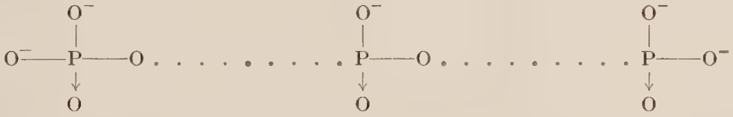
Reçu le 1 octobre 1957

L'ATTENTION des biochimistes est depuis quelque temps attirée par la préence dans divers organismes vivants de polyphosphates inorganiques. Ces omposés font partie d'un vaste groupe de substances, appelées phosphates ondensés, résultant de la combinaison entre elles de plusieures molécules 'orthophosphate. On distingue essentiellement deux groupes de phosphates ondensés :

(1) les polyphosphates, de formule générale $P_nO_{(3n+1)}M_{(n+2)}$, caractérisés ar une structure linéaire :

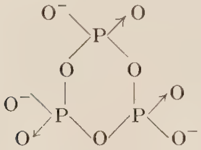


$$O^{-}\!-\!\overset{O^{-}}{\underset{O}{P}}\!-\!O\ \cdots\cdots\ \overset{O^{-}}{\underset{O}{P}}\!-\!O\ \cdots\cdots\ \overset{O^{-}}{\underset{O}{P}}\!-\!O^{-}$$

ls que le pyrophosphate ($n=2$), le tripolyphosphate ($n=3$), le tétrapolyhosphate ($n=4$), etc.

On connaît également des polyphosphates de longue chaîne, tels que le el de Graham, où n peut être égal à plusieurs centaines. Leur formule obale se rapproche de celle des métaphosphates cycliques $(PO_3M)_n$, au ur et mesure que n augmente dans la formule des polyphosphates. C'est a raison pour laquelle on les appelle souvent « métaphosphates », mais e terme tend à être réservé aux vrais métaphosphates cycliques.

(2) les métaphosphates, de formule $(PO_3M)_n$, caractérisés par une structure yclique, tels que le trimétaphosphate, le tétramétaphosphate, etc.



trimétaphosphate.

La présence de polyphosphates a été signalée dans un assez grand nombre d'organismes vivants : bactéries, levures, moisissures, algues [10]. Par contre la présence de métaphosphates cycliques n'a été signalée que dans un seul cas, dans un extrait de levure, où Kornberg [7] a signalé la présence de trimétaphosphate. Mais son existence *in vivo* est contestée [3].

Lorsque l'on étudie la répartition des polyphosphates dans divers organismes vivants, on constate que leur présence semble liée à la présence des corpuscules métachromatiques ou grains de volutine. Ces granulations sont caractérisées par une coloration « métachromatique » pourpre avec certains colorants basiques comme le bleu de toluidine. Cette coloration résiste à l'action de solutions acides diluées, qui font par contre disparaître la coloration « orthochromatique » des acides nucléiques.

La nature exacte de la volutine ou métachromatine est encore actuellement discutée. On a très rapidement reconnu qu'il s'agissait d'une substance phosphorée. L'étude de ses propriétés de solubilité et de coloration avait amené Meyer [9] à la conclusion qu'il s'agissait d'un composé de nature nucléoprotéique. Mais les travaux de Wiame [13–15] devaient montrer qu'elle renfermait des « métaphosphates », c'est à dire des polyphosphates de longue chaîne, responsables des propriétés de métachromasie.

Certains auteurs ont conclu à la présence simultanée dans les corpuscules métachromatiques d'acides nucléiques et de polyphosphates. C'est ainsi que Bringmann [1, 2] a constaté que les granulations de *Corynebacterium diphteriae* et de diverses autres bactéries présentaient également les colorations au vert de méthyle et à la pyronine, en général caractéristiques des acides désoxyribonucléiques et ribonucléiques. Mais ces observations doivent être interprétées avec beaucoup de prudence. En effet Stich [11] a montré que les polyphosphates dans *Acetabularia mediterranea* étaient intensément colorés par le vert de méthyle et la pyronine.

Ces résultats nous ont incités à effectuer une étude critique des colorations précédentes : métachromasie, vert de méthyle et pyronine, en utilisant des polyphosphates bien définis, de longueur de chaîne connue. Il nous a en effet paru intéressant de caractériser la longueur de chaîne minima des polyphosphates à partir de laquelle ces colorations devenaient positives.

Nous avons complété ce travail en étudiant certains composés autres que les phosphates condensés, susceptibles de donner des réactions positives avec les colorants précédents, à savoir divers esters phosphoriques (phytine) et sulfuriques (héparine, acide chondroïtine sulfurique, agar) de polysaccharides.

Composés étudiés

Polyphosphates.—Pyrophosphate de sodium : $P_2O_7Na_4$, $10H_2O$ (R. P. Prolabo). Tripolyphosphate de sodium : $P_3O_{10}Na_5$, $6H_2O$ (Monsanto Chemical Company). Sel de Graham (polyphosphate de longue chaîne) : préparé par chauffage à 900° du phosphate monosodique et refroidissement brusque. L'analyse chromatographique a montré que 90 % du produit étaient constitués par des polyphosphates de longue chaîne, le reste étant constitué par du trimétaphosphate, du pyrophosphate et de petites quantités de polyphosphates intermédiaires. La longeur de chaîne moyenne des polyphosphates était de 22 groupements PO_3.

Métaphosphates. –Trimétaphosphate de sodium : $P_3O_9Na_3$, $6H_2O$ (Monsanto Chemical Company). Tétramétaphosphate de sodium : $P_4O_{10}Na_4$, $4H_2O$ (Monsanto Chemical Company).

Produit d'hydrolyse du sel de Graham.—Nous avons également employé un échantillon de sel de Graham qui avait été soumis à une hydrolyse partielle acide. On obtient dans ces conditions un mélange de polyphosphates compris entre le pyrophosphate et le décapolyphosphate (tri-, tétra-, penta-, hexa-polyphosphates, etc.), de polyphosphates de plus longue chaîne et de métaphosphates (triméta-, tétramétaphosphates et métaphosphates de cycle plus large [12]).

Autres composés étudiés.—Phytine (inositohexaphosphate de Ca et de Mg); héparine (Hoffmann-Laroche); chondroïtine sulfate : échantillon préparé par M. Fauré-Frémiet à partir du cartilage de poisson.

Etude de la réaction de métachromasie

Des gouttes de solutions à 1 % des divers phosphates condensés et des autres composés ont été déposées sur une feuille de papier filtre que l'on a immergée dans une solution de bleu de toluidine à 1 ‰ dans l'eau. On a ensuite différencié par traitement par SO_4H_2 à 1 % : les taches des composés métachromatiques apparaissent en rouge violacé sur un fond légèrement bleuté.

Dans une autre expérience nous avons soumis à une séparation par chromatographie sur papier le mélange de phosphates condensés obtenu par hydrolyse partielle du sel de Graham. Cette technique permet de séparer les polyphosphates linéaires entre le pyrophosphate et le décaphosphate et l'ensemble des métaphosphates cycliques (triméta-, tétramétaphosphates, etc. [4–5]). On a effectué une chromatographie bidimensionelle avec les solvants suivants [4–5] :

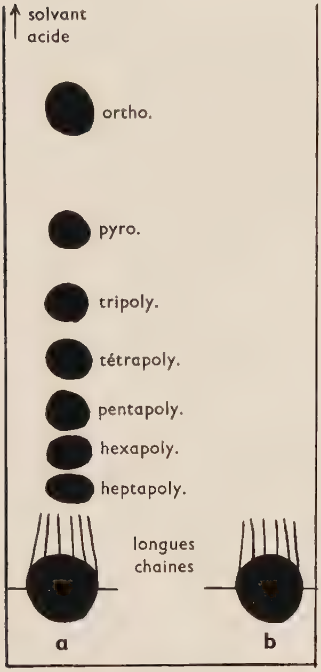


Fig. 1.—Chromatographie unidimensionnelle d'un mélange de polyphosphates. (*a*) Révélation par la technique de Hanes et Isherwood; (*b*) Révélation par la technique métachromatique.

(*a*) Solvant alcalin : 40 ml isopropanol; 20 ml isobutanol; 39 ml eau distillée et 1 ml ammoniaque (20% NH_3).

(*b*) Solvant acide : 70 ml isopropanol; 30 ml eau distillée; 4 g acide trichloracétique et 0,4 ml ammoniaque (20% NH_3).

Les divers poly- et métaphosphates ainsi séparés ont été révélés par l'une des deux techniques suivantes :

Technique de Hanes et Isherwood [6].—Cette technique qui révèle la totalité des poly- et métaphosphates, consiste à hydrolyser ces derniers en orthophosphate que l'on transforme en phosphomolybdate, réduit à son tour en bleu de molybdène :

On pulvérise sur le chromatogramme le réactif suivant : 5 ml acide perchlorique à 60%; 10 ml HCl *N*; 25 ml molybdate d'ammonium 4%; et eau distillée compléter à 100 ml. On sèche ensuite à l'étuve à 80° et on irradie aux rayons ultraviolets : les taches apparaissent en bleu sur un fond blanc.

Technique métachromatique.—Un autre chromatogramme identique au précédent a été traité par le bleu de toluidine et SO_4H_2 dans les mêmes conditions que précédemment : l'emplacement des phosphates condensés métachromatiques se manifeste par l'apparition d'une tache rouge violacée.

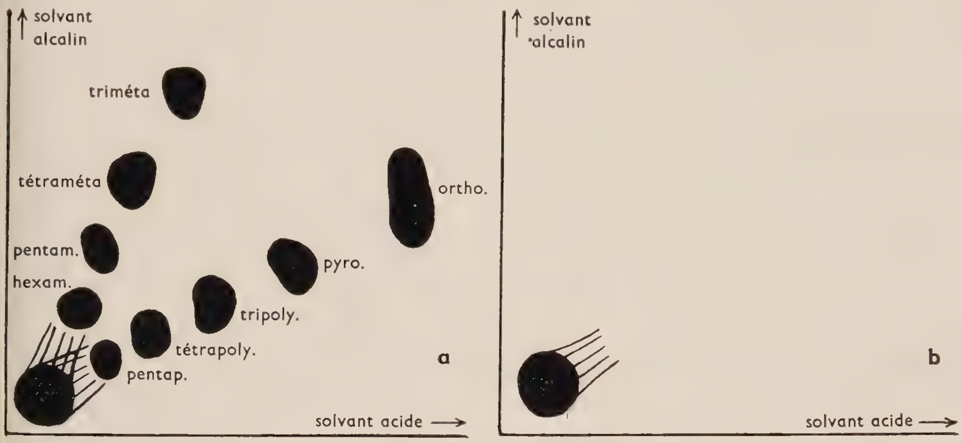


Fig. 2.—Chromatographie bidimensionnelle d'un mélange de poly- et métaphosphates. (*a*) Révélation par la technique de Hanes et Isherwood; (*b*) Révélation par la technique métachromatique.

Ces deux chromatogrammes révélés par les deux techniques précédentes ont été comparés entre eux. Les images obtenues (Figs. 1 et 2) montrent que :

(1) les métaphosphates cycliques ne sont pas métachromatiques;

(2) les polyphosphates linéaires sont métachromatiques à partir d'une longueur de chaîne de 8 groupements PO_3, c'est à dire à partir de l'octaphosphate.

Par ailleurs l'étude des produits autres que les phosphates condensés a montré qu'avec la phytine une coloration métachromatique apparaissait lors du traitement par le bleu de toluidine, mais qu'elle disparaissait lors de la différenciation par la solution acide.

On sait par ailleurs que les esters sulfuriques de polysaccharides : héparine, chondroïtine sulfate, agar, etc., sont métachromatiques [8].

La spécificité de la réaction de métachromasie est résumée dans le Tableau I.

TABLEAU I.

	Métachromasie	Vert de méthyle	Pyronine
Orthophosphate	–	–	–
Pyrophosphate	–	–	–
Tripolyphosphate	–	faible	faible
Polyphosphates $4 < n < 7$	–	faible	faible
Polyphosphates $n > 8$	+	+	+
Trimétaphosphate	–	–	–
Tétramétaphosphate	–	–	–
Phytine	–	–	–
Héparine	+	+	+
Chondroïtinesulfate	+	+	+
Agar	+	–	–

Etude de la coloration au vert de méthyle et à la pyronine

On a opéré comme précédemment, mais en immergeant les feuilles de papier filtre ou les chromatogrammes, soit dans une solution à 1 % de vert de méthyle dans l'eau ou dans l'acide acétique à 50 %, soit dans une solution à 1 % de pyronine dans l'eau.

Les résultats, résumés dans le Tableau I, montrent que :

(1) les métaphosphates cycliques ne sont colorés ni par le vert de méthyle, ni par la pyronine;

(2) dans le groupe des polyphosphates, une réaction faiblement positive apparaît avec les deux colorants à partir du tripolyphosphate, mais la coloration ne devient franche qu'avec les polyphosphates de longue chaîne à partir d'un degré de condensation voisin de 8 (octaphosphate).

On constate en outre que la phytine n'est pas colorée par ces réactifs, tandis que l'héparine et l'acide chondroïtine sulfurique, conformément à des observations antérieures (voir Lison [8]), donnent une réaction positive.

DISCUSSION

Le présent travail montre que, dans le groupe des phosphates condensés, seuls les polyphosphates de longue chaîne sont métachromatiques. Cette propriété apparaît avec un degré de condensation égal à 8 (octapolyphosphate).

Les polyphosphates de longue chaîne semblent d'ailleurs être les seuls composés phosphorés à présenter le phénomène de métachromasie, puisque la phytine (ester phosphorique de l'inositol) perd sa coloration lors de la différenciation par les solutions acides diluées et que, comme on le sait [8], les acides nucléiques ne sont pas métachromatiques dans les conditions ordinaires d'étude.

Le phénomène de métachromasie n'est cependant pas spécifique des polyphosphates de longue chaîne, puisque l'on sait que des polysaccharides macromoléculaires renfermant des radicaux esters sulfuriques (acide chondroïtine et mucoïtine sulfuriques, héparine, agar, etc.) et carboniques (gomme arabique, acide hyaluronique, etc.) (voir Lison [8]) présentent la même propriété.

Les polyphosphates de longue chaîne prennent également, comme les acides nucléiques et certains polysaccharides acides (héparine, acide chondroïtine sulfurique, etc.) la coloration au vert de méthyle et à la pyronine. Cette coloration apparaît au niveau du tripolyphosphate mais ne devient intense qu'à partir de l'octapolyphosphate.

Ces faits montrent que lorsque des corpuscules métachromatiques prennent la coloration au vert de méthyle et à la pyronine, ceci ne signifie pas nécessairement la présence d'acides nucléiques, puisque les polyphosphates de longue chaîne présentent eux-mêmes cette propriété.

RÉSUMÉ

Nous avons effectué une étude systématique des réactions de métachromasie et de coloration au vert de méthyle et à la pyronine appliquées aux phosphates condensés (poly-et métaphosphates).

Seuls les polyphosphates de longue chaîne à partir du degré de condensation 8 (octapolyphosphate) sont métachromatiques.

Les polyphosphates prennent également les colorations au vert de méthyle et à la pyronine. Cette propriété apparaît au niveau du tripolyphosphate, mais ne devient intense qu'à partir de l'octapolyphosphate.

Ces propriétés ont été comparées à celles d'autres composés phosphorés (acides nucléiques, phytine) et à celles des polysaccharides acides (héparine, chondroïtine sulfate, etc.).

SUMMARY

Metachromatic reaction and methyl green–pyronine staining were applied on condensed phosphates (poly- and metaphosphates) in a systematic study.

Metachromasia was obtained on long chain polyphosphates only (from the octapolyphosphate on).

Polyphosphates stain also with methyl green as well as pyronine. These staining reactions, which appear slightly with the tripolyphosphate, become intensive with a higher degree of condensation (octapolyphosphate).

A comparison was made with the behavior of other phosphorus compounds (nucleic acids, phytin) and with acid polysaccharides (heparin, chondroitine sulfate, etc.).

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II. MISE AU POINT DE MÉTHODES DE DÉTECTION CYTOCHIMIQUES SPÉCIFIQUES DES POLYPHOSPHATES

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Dans la note précédente [5], nous avons étudié les limites de spécificité de la réaction de métachromasie. Nous avons montré qu'à l'intérieur du groupe des phosphates condensés seuls les polyphosphates de longue chaîne supérieurs à l'octaphosphate présentaient cette propriété. On sait par ailleurs [8] qu'elle existe également chez divers esters sulfuriques et carboniques de polysaccharides macromoléculaires (héparine, acides chondroïtine et mucoïtine sulfuriques, acide hyaluronique, etc.).

L'absence de spécificité de la réaction métachromatique nous a conduits à la recherche de réactions permettant de différencier polyphosphates et polysaccharides acides. Stich [12] a constaté qu'après action de l'acide trichloracétique froid à 10% la coloration métachromatique disparaît, contrairement à celle des polysaccharides. Cette absence de métachromasie est due à l'extraction des polyphosphates par l'acide trichloracétique. Mais Wiame [14] a montré que les polyphosphates n'étaient pas totalement extraits par l'acide trichloroacétique. Il reste une fraction « métaphosphate » acido-insoluble constituée par des polyphosphates de très longue chaîne [7].

Cette réaction différentielle risque par conséquent de ne pas être tout à fait fidèle. Ce fait nous a amenés à rechercher une méthode de détection des polyphosphates strictement spécifique de ceux-ci. Plusieurs auteurs [1, 9, 13] avaient constaté que les corpuscules métachromatiques de *Corynebacterium diphteriae* pouvaient être mis en évidence par action des sels de plomb ou de cobalt suivie de celle du sulfure d'ammonium, selon une technique comparable à celle de Gomori pour la détection du phosphate libéré par action des phosphatases. Mais ces réactions ne présentent aucun caractère de spécificité sous la forme proposée par leurs auteurs [2]. Elles nous ont cependant incités à utiliser leur principe pour la mise au point de nos propres techniques [3, 4].

PARTIE EXPÉRIMENTALE

Reactifs employés

Nous nous sommes adressés à diverses réactions analytiques des polyphosphates que nous nous sommes efforcés d'adapter à la cytologie. Parmi les nombreuses réactions que nous avons étudiées, nous avons retenu les réactions de précipitation suivantes [3, 4].

Réactions avec les sels de plomb.—Les polyphosphates donnent avec les sels de plomb des précipités insolubles. Cette réaction peut devenir spécifique des polyphosphates aux pH acides. Nous avons employé une solution de nitrate de plomb à 10% dans l'eau en présence de solutions tampon à divers pH acides.

Réactions avec diverses amines.—Divers composés organiques basiques forment avec les polyphosphates des combinaisons insolubles pouvant servir à leur caractérisation [24, 25]. Parmi ces réactifs la benzidine, la tolidine et la dianisidine en solution dans l'acide acétique ont été les plus aptes à une application cytologique. Nous avons employé des solutions à 1% de ces amines dans l'acide acétique à 10 ou 20%.

Méthodes de révélation

Les polyphosphates précipités par ces réactifs ont été révélés de la manière suivante :

Sels de plomb.—Les polyphosphates de plomb insolubles sont décomposés par les sulfures alcalins en sulfure de plomb noir. Les préparations sont plongées pendant une minute dans une solution à 50 % de sulfure d'ammonium, puis lavées à l'eau distillée : il apparaît une coloration noire. C'est le procédé classique utilisé dans la détection cytologique des diverses phosphatases (Gomori).

Amines.—Nous avons d'abord essayé de réaliser, sur les combinaisons insolubles que donnent ces amines avec les polyphosphates, les réactions classiques de diazotation et de copulation en milieu neutre ou acide (action du nitrite de sodium en milieu acétique suivie de celle du β-naphtol, par exemple [13]). Nous avons obtenu une coloration brun rouge avec la benzidine et la dianisidine et violette avec la tolidine.

Mais ce type de réaction est délicat et nous avons préféré la réaction suivante plus facile à réaliser et qui donne des résultats constants : les préparations sont plongées pendant une minute dans de l'eau de brome qui oxyde les amines combinées aux polyphosphates avec apparition d'une coloration brunâtre avec la benzidine et brun noire avec la tolidine et la dianisidine.

Spécificité des réactifs précédents en fonction de l'acidité

Pour étudier les conditions dans lesquelles les réactifs précédents pouvaient devenir spécifiques des polyphosphates, nous avons fait divers essais en déposant sur une feuille de papier filtre des gouttes de solutions à 1 % dans l'eau des divers produits à étudier (polyphosphates, métaphosphates et divers composés succeptibles d'interférer avec ces derniers (acide ribonucléique, phytine, sulfates, polysaccharides acides). Après séchage, le papier a été immergé dans les divers réactifs pendant 15 minutes, puis lavé abondamment à l'eau distillée ou dans des solutions acides jusqu'à disparition du réactif dans les eaux de lavage. Puis le papier a été traité pendant une minute par le réactif révélateur approprié (sulfure d'ammonium ou eau de brome) et lavé à l'eau distillée.

Réactifs au plomb.—Nous avons étudié les réactifs suivants : 1 vol. d'une solution de $(NO_3)_2Pb$ à 10 % dans l'eau + 1 vol. de tampon; 0,2 *M* acéto-acétique pour les pH supérieurs à 3,0; 0,2 *M* NO_3H NO_3K pour les pH inférieurs à 3,0. Les lavages ont été effectués dans tous les cas à l'eau distillée

TABLEAU I.

Réactifs	Plomb pH 3.5	Plomb pH 1.0	Benzidine			Tolidine			Diani-sidine
lutions de lavage:	eau	eau	eau	CH_3COOH 10 %	HCl *N*	eau	CH_3COOH 10 %	HCl *N*	eau
mposés phosphorés									
n métachromatiques									
Ortophosphate	+	–	+	–	–	–	–	–	–
Pyrophosphate	+	–	+	–	–	–	–	–	–
Tripolyphosphate	+	–	+	+	–	–	–	–	–
Polyphosphates $3 < n < 8$	+	–	+	+	–	+	–	–	+
Trimétaphosphate	–	–	+	–	–	–	–	–	
Tétramétaphosphate	–	–	+	+	–	+	–	–	–
Phytine	+	+	+	–	–	+	–	–	–
A. R. N.	–	–	–	–	–	+	–	–	–
tachromatiques									
Polyphosphates $n > 8$	+	+	+	+	+	+	+	+	+
mposés non phosphorés									
1 métachromatiques									
Sulfates	+	+	+	+	–	+	+	–	–
tachromatiques									
Héparine	–	–	+	+	–	+	+	–	+
Acide chondroïtine sulfurique	–	–	–	–	–	–	–	–	–
Agar	–	–	–	–	–	–	–	–	–

usqu'à disparition du plomb dans les eaux de lavage. Les résultats sont ésumés dans le Tableau I.

, *Réactifs à base de benzidine, tolidine et dianisidine.*—Nous avons étudié liverses compositions de réactifs parmi lesquelles nous avons retenu les ormules suivantes : 1% benzidine dans 10% CH_3COOH; 1% tolidine dans 0% CH_3COOH; et 1% dianisidine dans 10% CH_3COOH.

Les lavages ont été effectués soit à l'eau distillée soit avec des solutions l'acide acétique ou chlorhydrique de concentration croissante. Les résultats ont résumés dans le Tableau I.

Contrôle des réactifs précédents avec des organismes renfermant des polyphosphates

Le Tableau I montre que l'on dispose de plusieurs réactifs dont la spécificité par rapport aux polyphosphates est suffisante pour conclure raisonnablement à la présence de ces composés dans la préparation étudiée. Il convenait cependant de contrôler la valeur de ces réactifs avec des organismes où la présence de polyphosphates avait déjà été démontrée par des méthodes chimiques.

Organismes étudiés

Nous avons effectué des essais sur les espèces suivantes :

Saccharomyces cerevisiae [2, 14].—Des levures de boulangerie ont été mises en suspension pendant une nuit avec aération intense dans un milieu synthétique dépourvu de phosphates minéraux. Dans ces conditions elles sont appauvries en phosphore et dépourvues de corpuscules métachromatiques. Elles sont ensuite cultivées avec aération dans un milieu dépourvu de composés azotés renfermant uniquement du glucose et du phosphate monopotassique. Dans ces conditions les levures synthétisent des quantités importantes de polyphosphates : au bout de 15 minutes on observe déjà une accumulation importante de corpuscules métachromatiques [14].

Aspergillus niger [6, 10], *Corynebacterium diphteriae* [2] et *Chlorella* [11, 15]. —Ces trois derniers organismes ont été étudiés dans des conditions physiologiques normales.

Fixateurs employés

Le matériel a été fixé pendant une minute aux vapeurs osmiques. Nous avons constaté que ce mode de fixation était celui qui conservait le mieux l'intégrité des corpuscules métachromatiques. Avec d'autres fixateurs (formol à 10%, fixateur de Helly, alcool), on observe soit une diffusion, soit une disparition de la substance métachromatique.

Description des techniques cytologiques

Métachromasie.—Les préparations fixées sont immergées 5 minutes dans une solution à 1 ‰ de bleu de toluidine, différenciées par passage pendant 15 minutes dans une solution d'acide sulfurique à 1% et montées dans l'huile de cèdre.

Réactif au plomb.—Les préparations fixées sont immergées dans les solutions de nitrate de plomb tamponnées précédentes pendant 15 minutes, puis lavées abondamment à l'eau distillée pour éliminer toute trace de sel de plomb. Elles sont ensuite traitées pendant une demi-minute dans une solution de sulfure d'ammonium à 50% et relavées à l'eau distillée.

Réactifs aux amines (benzidine, tolidine et dianisidine).—Les préparations fixées sont immergées pendant 15 minutes dans les solutions acides précédentes. Après lavage prolongé à l'eau distillée et aux solutions acides, elles sont traitées pendant une minute à l'eau de brome fraîchement préparée et ensuite relavées.

Résultats

Les réactifs précédents ont en général donné avec les organismes précédents des images se superposant aux corpuscules métachromatiques. Les résultats les plus nets ont été obtenus avec les réactifs suivants :

Réactif au plomb à pH 3,5 avec lavage à l'eau :
1 vol. d'une solution de $(NO_3)_2Pb$ à 10% dans l'eau + 1 vol. de tampon acéto-acétique 0,2 *M* à pH 3,5.

Réactifs à 1% de benzidine, de tolidine ou de dianisidine dans 10% d'acide acétique avec lavage à l'eau.

Les photographies 1–7 montrent les images obtenues avec la levure et avec *Aspergillus niger*.

DISCUSSION ET CONCLUSION

Les réactifs décrits n'ont pas tous la même valeur, tant au point de vue de la qualité de l'image que de leur spécificité vis à vis des polyphosphates. Le réactif le plus fidèle et le plus sensible est le réactif au nitrate de plomb à pH 3,5 avec lavage à l'eau. C'est lui qui donne les images les plus fines : les contours des corpuscules de polyphosphates sont plus précis qu'avec la coloration métachromatique ou la coloration par les amines (Fig. 1–7).

C'est aussi le réctif le plus spécifique. En effet il donne des résultats positifs avec les polyphosphates, des résultats négatifs avec les esters sulfuriques de polysaccharides. Les seuls composés qui puissent interférer sont la phytine et les sulfates. Mais les sulfates ne sont pas métachromatiques et la métachromasie de la phytine disparaît après traitement acide. Il en résulte qu'une réaction positive au plomb à pH 3,5, associée à une réaction métachromatique positive, permet de conclure avec certitude à la présence de polyphosphates.

Les réactifs aux amines sont moins spécifiques et donnent également des images moins précises. En effet, employés dans les conditions précédentes (solution à 1% des amines dans l'acide acétique à 10%, lavage à l'eau), ils donnent une réaction non seulement avec les polyphosphates, mais également avec l'héparine, substance métachromatique comme les polyphosphates. Le Tableau I montre qu'en lavant les préparations sur papier filtre avec diverses solutions acides on peut obtenir des réactions spécifiques des polyphosphates de longue chaîne. Mais en appliquant ce lavage acide aux préparations de levures, nous avons constaté que les résultats devenaient incertains. Dans certains cas la coloration persiste, dans d'autres elle devient incertaine, dans d'autres enfin elle n'apparaît plus. Cette différence s'explique soit par la présence dans les cellules de polyphosphates de longueur de chaîne plus courte que l'échantillon synthétique de référence employé dans nos expériences témoins sur papier filtre, soit par une pénétration moins bonne des réactifs à l'intérieur des cellules. On ne peut en effet pas comparer avec certitude des expériences réalisées sur papier filtre et des expériences effectuées sur des cellules. Les différences peuvent enfin s'expliquer par une quantité de polyphosphates plus faible dans les cellules que sur le papier filtre. En effet les réactions avec les amines sont moins sensibles que la réaction métachromatique ou que la réaction au plomb.

Les réactifs aux amines ne doivent être considérés que comme des réactifs secondaires pouvant servir de confirmation aux précédents. Ils peuvent cependant servir à éliminer tout doute possible quant à la présence de phytine ou de sulfates : le réactif à 1 % de dianisidine dans l'acide acétique à 10 % avec lavage à l'eau donne des résultats négatifs avec ces composés.

RÉSUMÉ

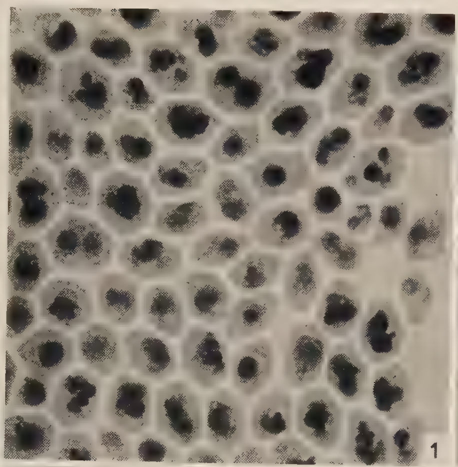
Nous avons mis au point une série de réactifs cytochimiques permettant de préciser la nature polyphosphorique des corpuscules métachromatiques.

Le réactif des polyphosphates inorganiques le plus spécifique et donnant les images les plus nettes est le nitrate de plomb à pH 3,5. Une réaction positive avec ce réactif, associée à une réaction métachromatique positive, permet de conclure avec certitude à la présence de polyphosphates.

Nous avons décrit d'autres réactifs à base d'amines (benzidine, tolidine et dianisidine). Mais moins spécifiques et donnant des résultats moins constants, ils ne doivent être considérés que comme des réactifs de confirmation.

SUMMARY

Several cytochemical reagents, allowing to determine the polyphosphoric nature of metachromatic bodies, were worked out.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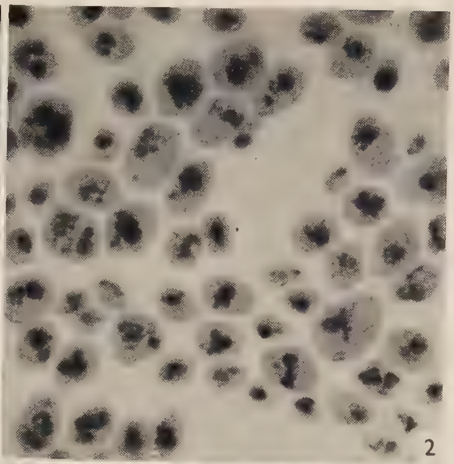
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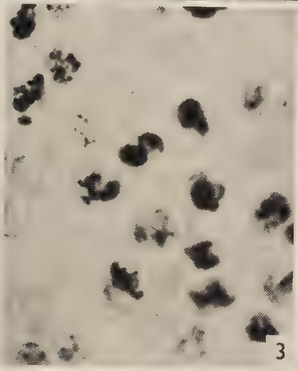
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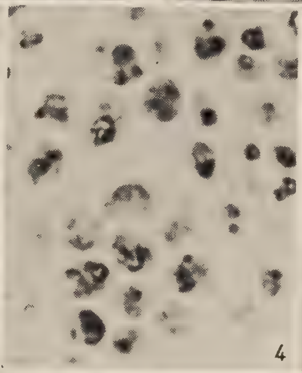
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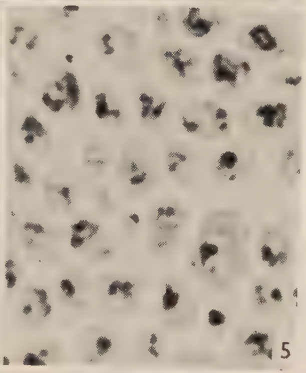
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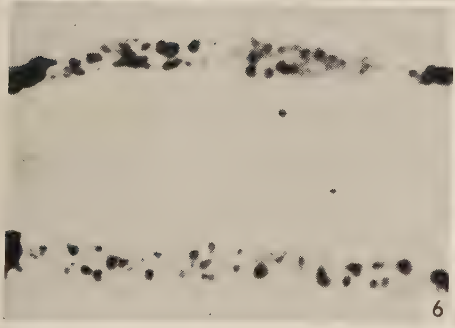
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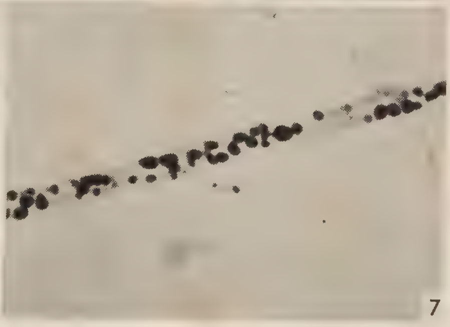
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Fig. 1.—*Saccharomyces cerevisiae*, Coloration métachromatique. 1500 ×.

Fig. 2.—*Saccharomyces cerevisiae*, coloration au plomb. 1500 ×.

Fig. 3.—*Saccharomyces cerevisiae*, coloration a la benzidine. 1200 ×.

Fig. 4.—*Saccharomyces cerevisiae*, coloration a la tolidine. 1200 ×.

Fig. 5. *Saccharomyces cerevisiae*, coloration a la dianisidine. 1200 ×.

Fig. 6.—*Aspergillus niger*, coloration métachromatique. 1300 ×.

Fig. 7.—*Aspergillus niger*, coloration au plomb. 1300 ×.

The most specific reagent of inorganic polyphosphates, which also gives the best pictures, is lead nitrate at pH 3,5. A positive result with this reagent, in association with a positive metachromatic reaction, allows to ascertain the presence of polyphosphates.

Other reagents containing amines (benzidine, tolidine and dianisidine) were described, but they are less specific and give less constant results.

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III. PRÉSENCE DE POLYPHOSPHATES CHEZ DIVERS ORGANISMES INFÉRIEURS

J. P. EBEL, J. COLAS et S. MULLER

Dans la note précédente [6], nous avons décrit la mise au point de réactions cytochimiques des polyphosphates inorganiques. Nous avons vérifié la valeur de ces méthodes en les appliquant à des organismes où la présence de polyphosphates avait déjà été démontrée par des méthodes chimiques. Nous avons constaté que l'on obtenait des images tout à fait comparables à celles données par la réaction de métachromasie.

Nous nous sommes proposés d'appliquer ces techniques nouvelles à divers organismes inférieurs où la présence de corpuscules métachromatiques était connue, mais où leur nature chimique n'avait pas été élucidée.

MATÉRIEL ET MÉTHODES

Notre étude a surtout porté sur des champignons inférieurs (Micromycètes) cultivés sur du moût de bière gélosé. Les filaments mycéliens ont été dilacérés, collés sur une lame par une mince couche de gélatine et fixés aux vapeurs osmiques. Comme réactifs nous avons utilisé essentiellement le réactif au nitrate de plomb tamponné à pH 3.5 spécifique des polyphosphates et dans de très nombreux cas les réactifs aux amines décrits précédemment.

Nous avons également fait quelques essais sur des algues et des protozoaires.

RÉSULTATS

La présence de polyphosphates inorganiques a été démontrée par les méthodes cytologiques précédentes chez les organismes suivants

Champignons.—Myxomycètes; Actinomycètes (*Streptomyces griseus*); Proascomycètes (*Rhodotorula rubra, Brettanomyces animalis, Candida vulgaris, Candida tropicalis, Mycotorula eisnerosa, Kloeckera Mulleri, Cryptococcus Laurentii* et *Torulospora rosei*); Plectascales (*Aspergillus niger* et *Aspergillus candidus*); Hypocréales (*Nectria rubi*); Sphaeriales (*Chaetomium globosum*); Discales (*Morchella hortensis*); Sphéropsidales (*Phoma hibernica* et *Diplodia natalensis*); Mélanconiales (*Colletotrichum Lindemuthianum* et *Myrothecium verrucaria*); Mucédinées (*Gliocladium roseum, Botryotrichum piluliferum* et *Staphylotrichum coccosporum*); Dématiées (*Curvularia maculans*); Stilbacées (*Isaria felina*); *Tuberculariées* (*Fusarium coeruleum*); *Mycelia sterilia* (*Rhizoctonia solani*); *Fungi imperfecti* (*Verticillium Koningii*).

Algues.—Cyanophycées (*Oscillatoria*) et Chlorophycées (*Chlorella*).

Protozoaires.—Rhizopodes (*Amoeba*) et Flagellés (*Chilomonas*).

Les figures (1 à 7) montrent les images obtenues dans le cas de *Fusarium coeruleum, Gliocladium roseum, Oscillatoria, Amoeba.* Dans certains cas (*Myrothecium verrucaria, Gliocladium roseum, Fusarium coeruleum* etc.) cette démonstration cytochimique a été complétée par une étude chimique des phosphates condensés constitutifs de la substance métachromatique, qui fera l'objet d'une note ultérieure.

DISCUSSION

Notre travail montre que les polyphosphates inorganiques sont largement répandus chez de nombreux organismes inférieurs. Rappelons que la présence de ces phosphates condensés avait déja été démontrée chez un certain nombre de bactéries : *Corynebacterium diphteriae* [2, 5], *Mycobacterium avium* [2], *Mycobacterium smegmatis* [19], *Klebselia aerogenes* [4] (voir la revue générale de Winkler [20]), et de champignons : *Aspergillus niger* [11, 15], *Saccharomyces cerevisiae* [5, 18], etc. Notre étude montre l'existence quasi universelle des polyphosphates chez les champignons inférieurs.

L'existence de polyphosphates avait également été signalée dans diverses algues : chez des Cyanophycées : *Phormidium* [5] et chez des Chlorophycées (*Chlorella* [16, 21], *Euglena* [1], *Acetabularia* [17]). Nous montrons dans le présent travail que les Oscillaires renferment également des corpuscules métachromatiques de nature polyphosphorique. Au cours de la rédaction de notre travail nous avons eu connaissance d'une note en préparation de Keck et Stich [12] qui ont étudié la présence de polyphosphates essentiellement dans des algues et des mousses et ont trouvé ces phosphates condensés dans les organismes suivants :

Myxomycètes (*Physarium*); Cyanophycées (*Oscillatoria*); Chrysophycées (Bacillariophycées : Fragelaria, Navicula et Hétérochontées : Vaucheria); Chlorophycées (*Ulothrix, Eudogonium, Zygnema, Mougeotia, Spirogyra, Cosmarium, Chara*); et Bryophytes (*Polytrichum, Leptobryum*).

Ce travail vient donc utilement compléter le nôtre.

Notre étude se termine enfin par une rapide investigation dans le règne animal où nous avons décelé cytochimiquement la présence de polyphosphates chez un Rhizopode (l'Amibe), et chez un Flagellé (*Chilomonas*). Des corpuscules réfringents de nature phosphorique ont été décrits récemment chez l'Amibe [7]. Il s'agit (Fig. 7) très vraisemblablement de polyphosphates et nous nous proposons d'en donner une démonstration purement chimique.

La présence de quantités importantes de polyphosphates dans un grand nombre de cellules a suscité plusieurs travaux sur leur synthèse et leur rôle métabolique. Leur synthèse nécessite la présence de processus cataboliques fournissant de l'énergie, comme la fermentation ou l'oxydation [18], qui entrainent la formation de liaisons riches en énergie sous forme d'ATP.

Fig. 1.—*Gliocladium roseum,* coloration métachromatique. 2400 ×.

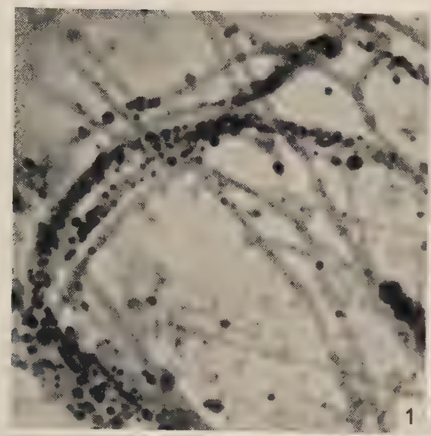
Fig. 2.—*Gliocladium roseum,* coloration au plomb. 2400 ×.

Fig. 3.—*Fusarium coeruleum,* coloration métachromatique. 2400 ×.

Fig. 4.—*Fusarium coeruleum,* coloration au plomb. 2400 ×.

Fig. 5.—*Oscillatoria* species, coloration métachromatique. 750 ×.

Fig. 6.—*Oscillatoria species,* coloration au plomb. 750 ×.



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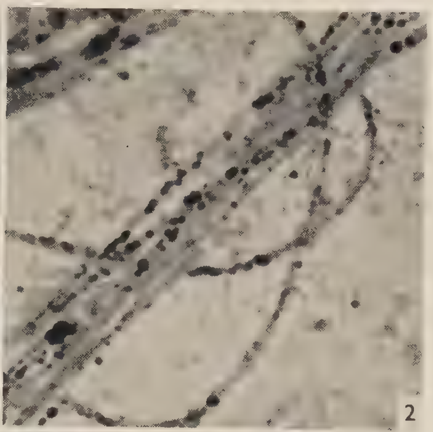

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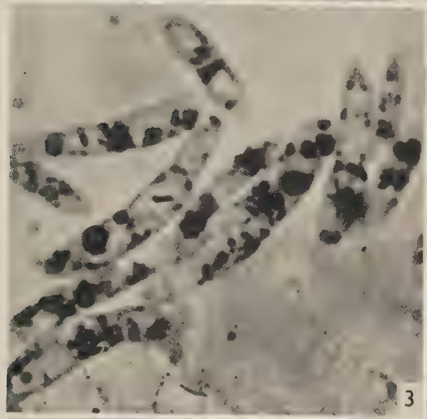

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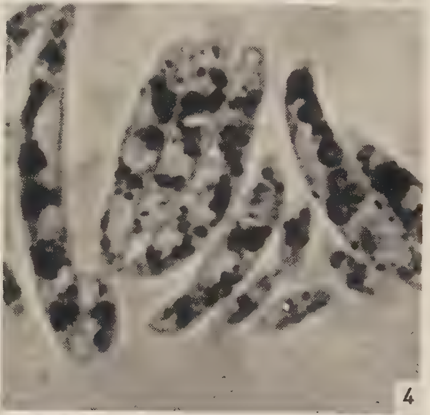

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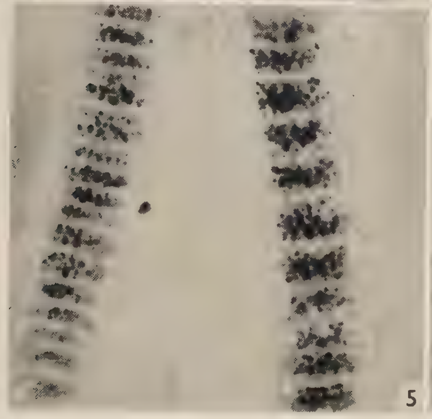

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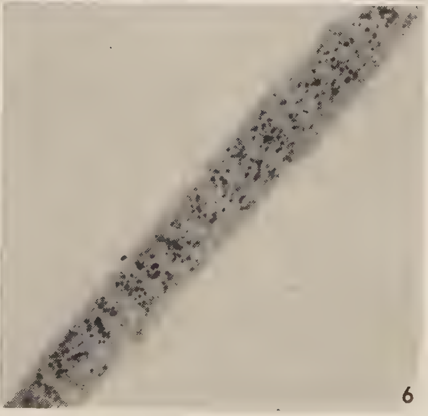

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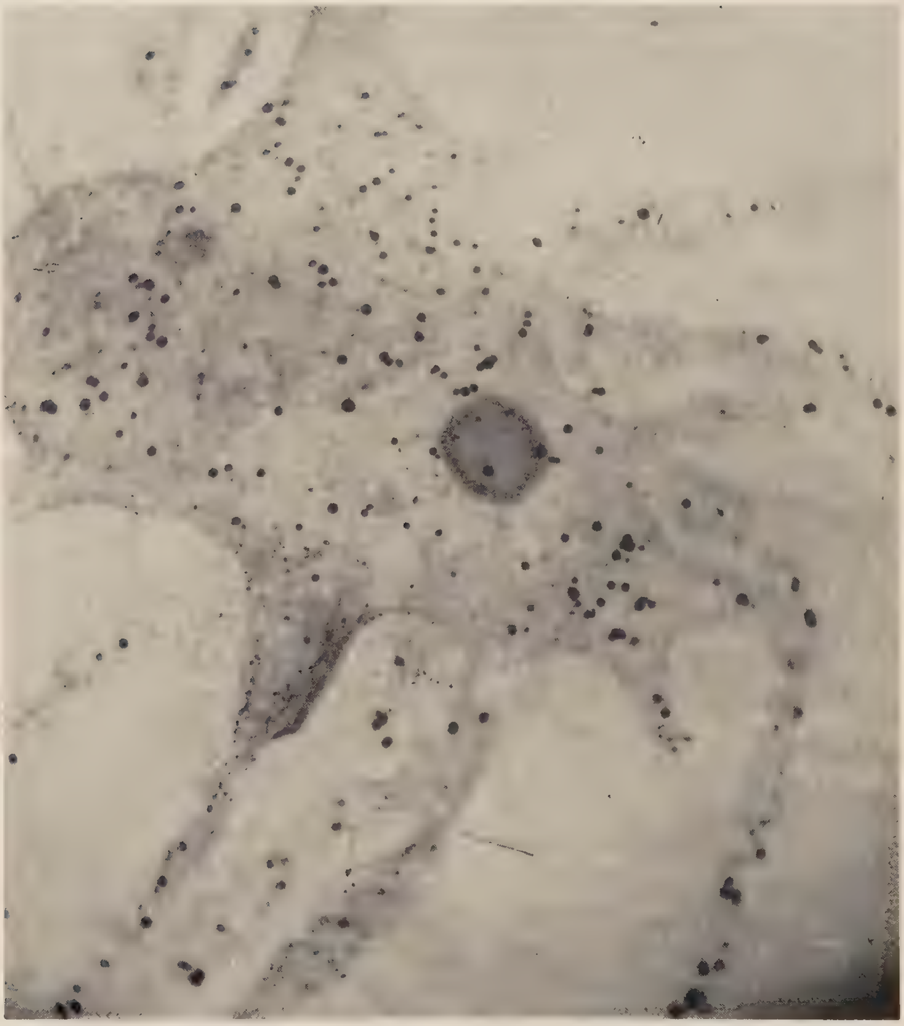

Fig. 7.—*Amoeba proteus*, coloration au plomb. 500 ×.

Des systèmes enzymatiques catalysant la formation de polyphosphates à partir de l'ATP et la réaction inverse :

$$\mathrm{ATP} + (\mathrm{PO}_3^-)_n \rightleftharpoons \mathrm{ADP} + (\mathrm{PO}_3^-)_{n+1}$$

ont été mis en évidence dans la levure et *Escherichia coli* [9, 13, 14].

L'accumulation des polyphosphates dans la cellule est favorisée par toute une série de facteurs défavorables à la croissance et à la multiplication cellulaire. Ce fait suggère que la synthèse des polyphosphates est liée à une absence d'utilisation de l'ATP pour la synthèse des protéines ou des acides nucléiques ou pour d'autres processus anaboliques [19]. Une fonction énergétique analogue à celle des phosphagènes chez les animaux a été suggérée [8], mais ce rôle des polyphosphates a été remis en question par son auteur même [10]. Signalons enfin la propriété des polyphosphates d'inhiber toute une série de réactions enzymatiques [3].

Le problème se pose de l'existence de ces phosphates condensés inorganiques chez des animaux supérieurs. Une accumulation de tels composés, comparable à celle que l'on observe chez les organismes inférieurs, semble extrèmement peu probable, mais la présence de polyphosphates inorganiques en très faibles quantités non détectables cytochimiquement, ne doit pas être écartée, la formation enzymatique du pyro- et du tripolyphosphate ayant été démontrée dans de nombreuses réactions *in vitro*.

RÉSUMÉ

Nous avons démontré par des techniques cytochimiques la présence de polyphosphates inorganiques de longue chaîne dans un grand nombre d'organismes inférieurs : champignons, algues et protozoaires.

Nous avons passé en revue les connaissances actuelles sur la répartition des polyphosphates dans l'ensemble des organismes vivants et sur leur formation et leur rôle physiologique.

SUMMARY

The presence of inorganic long chain polyphosphates in numerous lower organisms (molds, algae, protozoa) was demonstrated with cytochemical technics. A review is made of the present knowledge concerning the distribution of polyphosphates in living organisms, their biochemical formation as well as their physiological significance.

Nous adressons nos remerciements a Monsieur Fauré-Frémiet pour l'intéret qu'il a porté à ce travail et pour les conseils qu'il nous a prodigués.

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NUCLEIC ACIDS AND THEIR COMPONENTS AS AFFECTED BY THE Y CHROMOSOME OF *DROSOPHILA MELANOGASTER*

I. CONSTITUTION AND AMOUNT OF THE RIBONUCLEIC ACIDS IN THE UNFERTILIZED EGG[1]

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Received October 3, 1957

The importance of the ribonucleic acids in the biosynthesis of the proteins, first deduced on cytochemical grounds, is now generally accepted by biochemists. A hypothesis currently favored involves the assembly of amino acid residues on a ribonucleic acid templet, whose form depends on the structure of the nucleic acid. In these terms the genetic control of the composition of the ribonucleic acids possesses more than its own intrinsic interest. The heterochromatic regions of the chromosomes have been postulated for some time as having a role in the genetic control of nucleic acid synthesis; the present series of papers provides chemical evidence for this postulate.

The Y chromosome of *Drosophila melanogaster* has long been the representative case of an almost completely heterochromatic chromosome, in that (1) it forms characteristic blocks of deoxyribonucleic acid (DNA) in the interphase nuclei of mitotically active cells, and (2) very few mutations have been cytologically localized in these regions in comparison to the number in euchromatic regions of comparable metaphase length. An augmentation of the nucleic acid content of the cytoplasm of the *Drosophila* oocyte by the Y chromosome was one of the basic facts [7] in theories relating the heterochromatic regions of the chromosomes to the general nucleic acid metabolism of the cell. These data were obtained by cytophotometric methods; they showed on the average an increment in the optical density of the cytoplasm at 257 mμ amounting to 15 per cent [34]. Further studies of this type are continuing in these laboratories. Independent chemical evidence of the effects of the Y chromosome on the egg cytoplasm was sought by Callan [6] who found no quantitative difference in the ribonucleic acid (RNA) content of developing eggs. However, neither his methods nor the uniformity of the egg population he studied were sufficiently precise to consider that the question had been settled (cf. [35]).

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[1] Aided by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; by a grant from the U.S. Public Health Service C1613; and by an institutional grant of the American Cancer Society.

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The present study attempts a chemical analysis of the RNA of two genetic types, XXY^{sc8} and XX, in the unfertilized *Drosophila* egg. Here standard populations of eggs can be obtained, in contrast to the situation in the fertilized egg where the extremely rapid mitoses after fertilization complicate the issue by possibly causing changes in nucleic acid metabolism. It should be emphasized that even this information is not comparable to the earlier studies of the oocyte in the process of egg formation, a point to which we will return in the discussion. However, the unfertilized egg stands at the crest of the divide between oogenesis and development, and for studies in either direction, forms a necessary point of reference.

In the comparison of the RNA's in different genetic types, a study of the total amounts of nucleic acid, only, is no longer adequate in the light of recent developments in nucleic acid chemistry; particularly at present when it is possible to determine the purine and pyrimidine composition of the nucleic acids by the methods of chromatography. It is well known for example, that different fractions of cell homogenates may contain RNA of different base composition. In this study, therefore, an estimate of the amounts of RNA in the different egg types was preceded by a determination of the base composition of their RNA. As will be seen, in the unfertilized egg the quantities of the nucleic acids show little difference—a point previously established for the mature salivary glands of the larva [30]. However, when the base compositions of the nucleic acids are compared, a difference between the two types of RNA becomes apparent, and establishes perhaps the first case in which the composition of a nucleic acid is altered by the genetic constitution of the individual.

Preliminary reports and a general discussion of these data have already been presented [15, 16, 34].

EXPERIMENTAL

Genetic strains

The collection of large quantities of unfertilized eggs was facilitated by the fact that these are laid in quantity by females mated to males which are sterile due to lack of the Y chromosome. These males can be easily obtained as progeny from normal Oregon R mothers mated to males containing the attached XY^{sc8} chromosome [17]. Daughters of this cross contain the attached XY^{sc8} chromosome, in addition to the

X received from the mother; the males contain only the X chromosome from their mother, receiving no Y from the father. By the mating of the XO males to their $X\overline{XY}^{sc8}$ sisters, large quantities of unfertilized eggs from mothers containing an extra Y chromosome are obtained. The attached Y, it should be stated here, is not strictly comparable to a normal free Y. It also contains in addition a duplicate heterochromatic region presumably from the X chromosome as well as a small duplication of genes, section 1A, at the left end of the X chromosome. For the normal mothers without the Y chromosome, a standard Oregon R strain inbred by brother-sister matings for over 100 generations was used. The attached $\overline{XY}^{sc8}$ strain had previously been made isogenic with the Oregon R with respect to the autosomes and, by crossovers to the left of the locus white and to the right of the locus forked, for most of the X chromosome. The unfertilized eggs were obtained either from the collection of virgins, or by the use of the technique of Novitski and Rush [26], in which fertilized females are de-seminated by cold treatment. These females were then mated to XO males as used with the $X\overline{XY}^{sc8}$ females.

Egg collections

Groups of two hundred and fifty to three hundred females with approximately twice that number of males were placed in cotton-stoppered quart Mason jars, each containing a stainless steel tray, 5 × 2 × $\frac{3}{8}$ inches in size. These trays were filled from a molasses-agar mixture, which consisted of 420 ml molasses, 35 ml moldex (10 per cent methyl parasept in ethanol), 2800 ml water and 70 g Bacto-agar, upon which two lumps of yeast approximately 1 cm in diameter were spaced. In this way, the females were fed adequately for as much as a 24-hour period and treated thus, continued to lay well for as long as two weeks.

For collection, the bottom of each jar was gently tapped on a rubber pad so that the flies fell to the bottom and, in one operation, the tray upon which the eggs were laid could be removed and a fresh one inserted. Since very little egg laying occurs during the first half hour after a new tray is placed in the jar, two hours appeared to be a minimum period for the laying of large numbers of eggs.

It is well known that on standing, the unfertilized *Drosophila* egg gradually undergoes an internal autolysis; the yolk platelets break down and a redistribution of material occurs. As Fig. 1 shows, this process has not yet begun to any great extent at 3 hours. It was, therefore, decided to adopt as a standard, a collection period of 3 hours which would provide eggs whose maximum age would be about 2½ hours. Also, in order to have a measure of what happens during autolysis, egg trays were removed from the jars after a 12-hour laying period and the eggs were allowed to age on the trays in a moist atmosphere for another 12 hours before they were collected. The minimum age of these eggs was thus 12 hours. These are the samples subsequently referred to as "24-hour" eggs.

The eggs were brushed off the molasses agar with a soft camel's hair brush into a Petri dish containing 70 per cent ethanol. By gently swirling the dish, the eggs were concentrated at the center of the dish and the adherent medium washed away. This process was repeated with fresh 70 per cent ethanol until all the contaminants were removed. Then, the eggs were transferred to a vial, containing a 3:1 ethanol-ether solution, which was immersed in a dry-ice acetone bath. After the eggs were frozen, they were stored at −40°C until required.

For more precise aging, samples of 125 eggs laid during a 30 minute period were collected and immediately extracted.

Egg counts

Depending on the number of eggs involved, three methods were used to count the collected eggs. The eggs in the 125 egg samples were counted as they were collected from the trays. When larger numbers of eggs of one type were required, the following method was used: The combined egg collections were removed from the deep freeze, washed in ether and dried in a vacuum desiccator overnight. The entire batch of eggs was weighed and the weight recorded. Then a small portion of eggs was removed, weighed, and the eggs were counted under a dissecting microscope. After several experiments of this kind, it was found that the average weight of a *Drosophila* egg dried in this manner was 4.56 μg.

The above method was satisfactory with large egg batches (more than 50,000 eggs); but when it was necessary to determine the number of eggs in a single 3-hour collection (approx. 1000–2000 eggs), the fact that the dried eggs were hygroscopic introduced a serious error. This was avoided by devising another method of counting. Before being transferred to a vial for freezing, the eggs were dispersed in a single layer just covered by 70 per cent ethanol over the bottom of the Petri dish. Then they were photographed against a dark background at 1 × magnification. The negative combined with the negative of a grid, was enlarged 3 × and printed. Meanwhile, the eggs were swirled again in 70 per cent ethanol, transferred to a 3:1 (*v*/*v*) ethanol-ether solution and put into a calibrated Bauer-Schenck centrifuge tube whose fine tip from 0–.05 ml had been filled with paraffin. The eggs were centrifuged for ten minutes at 2000 RPM and their volume measured. Finally, they were transferred to a vial and stored as described above.

The number of eggs was counted on the photograph. From the counts and the volumes of several different egg collections, it was found that one egg has a volume of $28.5 \pm 4 \times 10^{-7}$ ml. Thus, for subsequent egg collections the number of eggs could be found by measuring only their volume. The maximum error in this method is ± 5 per cent. The entire procedure, from the initial removal of the egg tray from the jar to the final freezing of the eggs, occupies twenty minutes or less. During this time the eggs undergo no apparent autolysis.

The three types of samples from these collections will be referred to as follows: (1) *125 egg samples*; (2) *small batches*, simultaneous 3-hour collections from several jars (1000–2000 eggs); (3) *large batches*, the aggregate of numerous small batches (more than 50,000 eggs).

Extraction procedures

Lipid materials.—The eggs which had been stored in the 3:1 ethanol-ether solution were subjected to a series of extractions, an ether extraction to remove lipid material, a 1 per cent cold perchloric acid extraction to remove, among other substances, cold acid-soluble nucleic acid components; and finally an extraction with hot sodium chloride or hot 9 per cent perchloric acid to remove the RNA.

Prior to ether extraction, the frozen eggs were washed with ether until none of the ethanol in the storage solution remained. The washings were decanted, combined

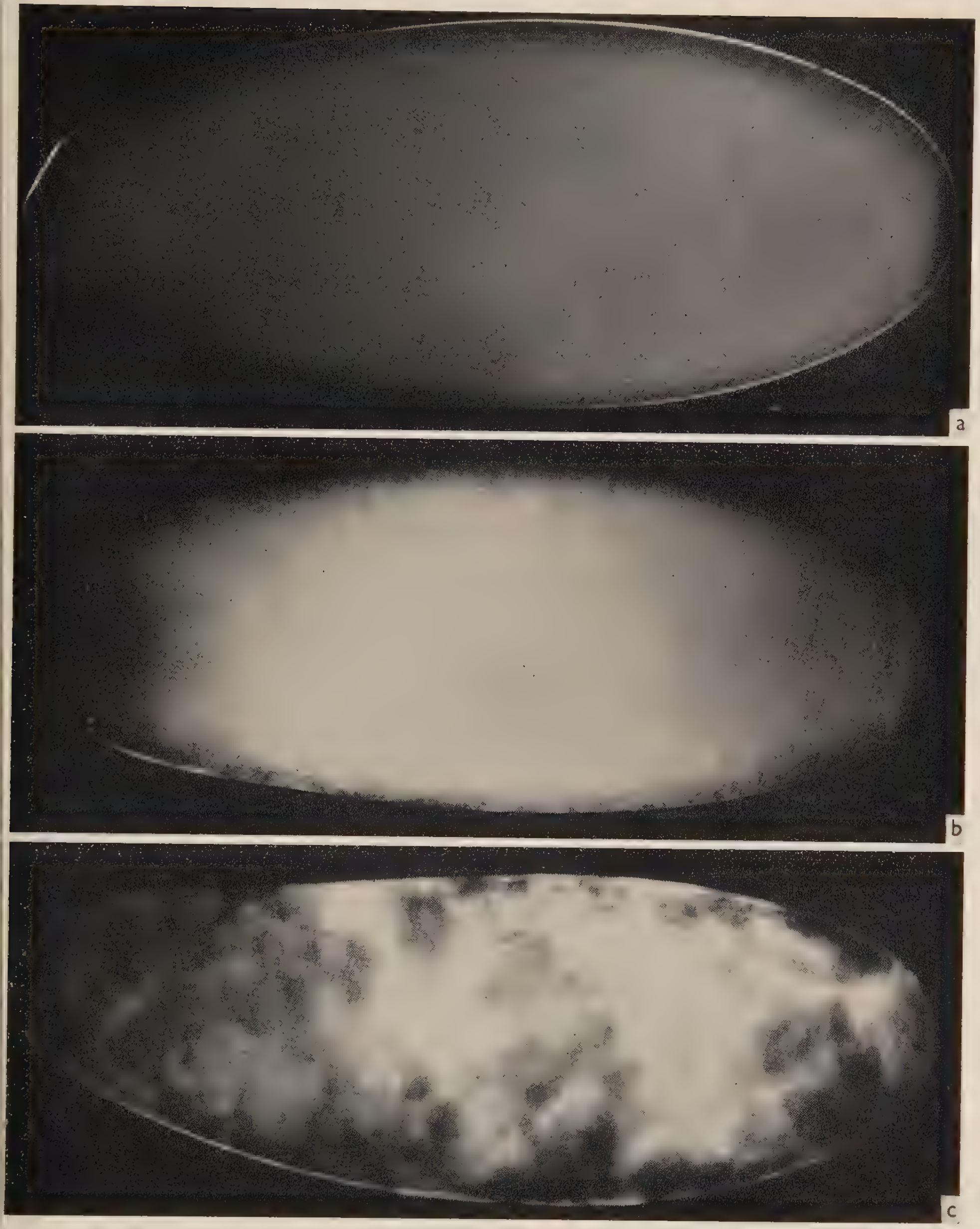

Fig. 1.—Unfertilized eggs of *D. melanogaster*: (*a*) immediately upon being laid; (*b*) 3 hours after being laid; and (*c*) 24 hours after being laid.

with the ethanol-ether storage solution and taken to dryness. The residue was taken up in boiling water and was tested for ultraviolet absorption, which was found to be negligible.

For the actual ether extraction, the large batches of ether-washed eggs were dried in a vacuum desiccator overnight and weighed, then homogenized in ether at room temperature. The homogenate was refluxed successively with ether for 4 hours, and petroleum ether for 2 hours; the respective supernatants were combined and evaporated to dryness. The lipid thus extracted was weighed and stored in the cold for possible future use.

The small egg batches were homogenized in 2 ml of ether and centrifuged; the treatment was repeated using first ethyl ether and then petroleum ether. These supernatants were discarded.

Since the demands of quantitation were more exacting with the 125 egg collections, they were extracted with ether in yet another manner. Directly after collection and counting, each group of 125 eggs was homogenized at room temperature in a modified micro, all glass Potter-Elvehjem homogenizer containing 1.0 ml of 3:1 ethanol-ether, and allowed to extract overnight. The suspension was centrifuged, the clear supernatant transferred to a 2.0 ml volumetric flask, and the extraction repeated twice with 0.5 ml volumes during the ensuing 24 hours. The pooled supernatants were finally adjusted to 2.0 ml with ethanol-ether and used for analyses to be described in the succeeding communication.

Cold acid soluble substances.—The dried lipid free residues were extracted with cold 1 per cent perchloric acid at 4°C [33]. The residues of the large egg batches were extracted twice with 10 ml volumes of 1 per cent perchloric acid for 5 minutes, with frequent stirring to prevent settling of the particulates. Following centrifugation (3 mins), the residue was washed once with 5 ml of cold distilled water, centrifuged, and the combined supernatants neutralized with 5 *N* ammonium hydroxide. The neutral solution was deproteinized by shaking with an equal volume of chloroform containing octyl alcohol [37] and repeating this treatment until no more protein was precipitated. This solution, subsequently termed the cold perchloric acid extract, was lyophilized, taken up in 1.0 ml of distilled water and stored in the cold. The residue was dried *in vacuo* and stored at −40°C.

The lipid free residues of the small egg batches were treated similarly, except that 2 ml aliquots of 1 per cent perchloric acid, and 1 ml of water were used. Also, it was found unnecessary to deproteinize the extract.

The residues of the 125 egg collections were also treated in a similar manner, except that instead of the 1 per cent perchloric acid, 0.3 ml volumes of 9 per cent (w/v) perchloric acid were used and the final volume of the supernatants was made up to 1 ml with 9 per cent perchloric acid and analyzed immediately instead of being neutralized and lyophilized.

In all cases described above the total period for the complete cold acid extraction did not exceed 26 minutes and a third acid treatment did not extract any detectable phosphorus or ultraviolet absorbing compounds.

Nucleic acids.—For preparation of purified RNA only the residues remaining after cold acid extraction of the large batches were employed. The nucleic acids were extracted with 25 volumes of 10 per cent (w/v) sodium chloride [9]. The extractions were performed on the steam bath with continuous stirring for 8 hours, during which period the insoluble material was centrifuged down three times, the supernatant decanted, and replaced with fresh sodium chloride solution; 2.5 volumes of 95 per cent ethanol were then added to the combined sodium chloride extracts, and the polymerized nucleic acids allowed to precipitate overnight in the refrigerator. Following centrifugation the nucleic acids were dissolved in 6.0 ml of 0.05 *M* sodium chloride [38] and re-precipitated with 2.5 volumes of ethanol overnight at 4°C. The re-precipitation treatment was repeated twice more, and the resulting sodium nucleates were washed successively with cold 70, 90 and 100 per cent ethanol and ether, and were subsequently air dried. These purified nucleic acids were used in the determination of the base constitution of the RNA from the two types of egg.

For quantitative evaluation, nucleic acids were extracted from the egg residues of the small egg batches by extracting twice with 1 ml portions of 9 per cent perchloric acid at 80°C for 10 minutes. The residual material was washed once with 0.5 ml of 9 per cent perchloric acid and centrifuged immediately. The supernatants were combined and analyzed. The residues of 125 egg collections were treated similarly except that 0.3 ml volumes of 9 per cent perchloric acid were used, and the combined supernatants were made up to 1 ml.

Chemical analyses

Base analysis.—The base composition of the hot salt extracted ribonucleic acids was determined on samples weighing 2 mg by two different methods. In the first of these the RNA was hydrolyzed in 0.3 *N* potassium hydroxide and treated according to the procedure of Crosbie, Smellie and Davidson [8]. The resulting ribonucleotides were separated by paper ionophoresis as described by Markham and Smith [22], and the individual nucleotides were localized on the paper with the aid of a General Electric 8 W germicidal lamp fitted with a Corning No. 9863 filter. The "spots" and appropriate blanks were cut out and eluted overnight in 2.0 ml of 0.1 *N* hydrochloric acid in stoppered tubes. The tubes were centrifuged and the absorption of the eluted nucleotides measured in quartz microcells in the Beckman Model DU spectrophotometer. It was found that the wavelengths of maximal absorption of the 4 nucleotides (ε max.) were almost identical with those quoted by Deluca, Rossiter and Strickland [10], whose values for the corresponding molar extinction coefficients were employed in calculating the nucleotide concentrations. At least two separate determinations in duplicate were performed on each sample of RNA, and any electrophoretic run in which duplicate nucleotides did not agree to within 5 per cent was rejected.

The total recovery of nucleotides relative to the phosphorus content of hydrolysate varied from 93–98 per cent in the various experiments.

The second method employed for the base analyses was that of Loring, Fairley, Bortner and Seagrave [19], whose directions for the determination of purines were followed exactly. The pyrimidine bases were not determined.

Determination of RNA content.—The RNA content of the eggs was determined by ultraviolet absorption and estimation of phosphorus and ribose.

Ultraviolet absorption spectra of the hot perchloric acid extracts were taken at pH 1 and 11. For use as a standard and for purposes of comparison, the same types of analyses were made of the purified RNA in aqueous solution at pH 7, 1 and 11;

and again when the dry sample was subjected to the conditions of the 9 per cent hot perchloric acid extraction.

Inorganic and total phosphorus analyses were made for each sample according to the method of Allen [2] employing a final valume of either 0.5, 1.0, or 2.0 ml as required.

Following upon the recommendation of Miller, Golder and Miller [25], ribose was determined according to Drury [12], using 0.2 ml aliquots of unknown made to a final volume of 1.05 ml and a heating time of 30 minutes.

Deoxyribose was measured on 0.2 ml aliquots of unknown (final volume 0.6 ml) by the Patterson and Dackerman [29] modification of Dische's method [11]. We are indebted to Dr. L. M. Koehler for a sample of pure deoxyribose which we employed as a standard.

For nitrogen estimations, the micro-Kjeldahl procedure was employed. With few exceptions, all chemical determinations were made in duplicate.

RESULTS

Constitution of the RNA's. -In this paper only the RNA in the different egg types will be considered. The numerous low molecular weight nucleic acid components of the cold acid extract will be described in a succeeding communication.

Preparations of nucleic acids made by the hot salt method, detailed above, served as a source of material for the analysis of the base composition of the nucleic acids. In such preparations, the DNA content is very low. The extent to which one $\overline{XX}Y^{sc8}$ sample was contaminated with DNA was determined by carrying out the diphenylamine reaction on 0.1 ml aliquots of an RNA solution containing 20.1 mg RNA/ml. The average concentration of DNA was 110 μg/ml indicating a contamination of DNA of approximately 0.5 per cent by weight. This small amount was well below the sensitivity of the methods employed in the succeeding analyses.

All the RNA preparations, however, were contaminated to a greater or lesser degree with glycogen. Although the presence of the polysaccharide did not interfere in the analyses of the purine and pyrimidine constituents of the nucleic acids when the method of alkaline hydrolysis was used, it should be mentioned that Marshak and Vogel's method of hydrolysis (72 per cent perchloric acid, 1 hour at 100°C) [23] could not be applied to this material. Excessive caramelization, probably due to breakdown of the glycogen, produced a copious carbon precipitate which was found differentially to absorb significant quantities of the bases.

The presence of ribose as the sole pentose, together with smaller amounts of glucose—undoubtedly derived from the contaminating glycogen—was demonstrated chromatographically on a sample of XXY^{sc8} and XX RNA as follows: The nucleic acid was hydrolyzed with 1 *N* hydrochloric acid for 60 minutes at 100°C, and after centrifugation the supernatant fluid was evaporated to dryness. The dried residue was extracted with pyridine and 10 per cent isopropanol as described by Malpress and Morrison [20] in preparation for ascending chromatography, employing either *n*-butanol-ammonia [21] or *n*-butanol-acetic acid [31] as solvents. The sugar "spots" as revealed by a number of procedures [5] coincided in position with those of known ribose and glucose markers.

The degree of purity of the RNA preparation is evidenced by the absence of a positive biuret test and by a molar nitrogen/phosphorus ratio of 3.9 for the XXY^{sc8} RNA (theoretical: 3.77). The molar purine ribose/phosphorus ratios were 0.58 and 0.54 for the XXY^{sc8} and XX types of RNA respectively. The corresponding theoretical values of these ratios calculated from the base analyses presented below are 0.53 and 0.50. The percentage of phosphorus in the different preparations was consistently low (5–7 per cent) probably owing to the presence of the contaminating glycogen.

It is well known [24] that the ratios of the constituent purines and pyrimidines may vary widely in different types of RNA. The determination of the base composition of the RNA from the two genetic types of eggs with which we are concerned was therefore taken as a point of departure in this study. Following alkaline hydrolysis and ionophoresis by the methods described, the individual mononucleotides were characterized by ultraviolet absorption spectra at pH's 1, 7 and 11; and by chromatographic and spectroscopic analyses of the bases resulting from their acid hydrolysis. The base composition of three different preparations of RNA from XX and XXY^{sc8} eggs is shown in Table I, the data being calculated as moles of base per 100 moles of phosphorus. It will be noted that in the XX type of eggs the purine to pyrimidine ratio is 0.97 whereas in the XXY^{sc8} type the ratio is 1.13. This difference is clearly due to an increase in the relative amount of adenylic acid in the XXY^{sc8} nucleic acid, an increase that is consistent for all three preparations.

A confirmation of this difference in the purine composition of the two types of nucleic acid was obtained by a chemical determinatjon of purines [19]. In this method, RNA is hydrolyzed in normal acid at 100°C and the liberated purines precipitated as the silver salts; the latter are then decomposed and the free purines determined by ultraviolet absorption at several wavelengths. Table II shows the results obtained by this procedure. The XXY^{sc8} type RNA contained on the average 0.494 moles of purine per mole of nucleotide phos-

TABLE I. *Purine and pyrimidine base distribution in RNA from two genetic types of* Drosophila melanogaster *eggs.**

(Moles of base/100 moles of phosphorus).

			Egg type									
			$X\overline{XY}^{sc8}$					XX				
Max. age (hrs.)	Prep. #	Sample #	A	G	C	U	Pu/Py	A	G	C	U	Pu/Py
3	1	a	29.7	24.1	18.1	28.0		23.6	25.4	20.0	31.0	
		b	29.9	23.1	18.7	28.5		24.0	25.2	20.6	30.4	
	2	a	29.8	25.0	18.3	27.2		24.2	25.4	20.5	29.9	
		b	30.1	23.6	18.4	27.7		24.2	24.5	19.5	31.9	
	3	a	29.5	22.4	19.0	29.5		27.9	23.0	20.8	28.1	
		b	29.5	21.9	18.4	30.1		26.9	22.8	21.5	29.0	
	Mean		29.8	23.1	18.5	28.5	1.13	25.2	24.4	20.5	30.0	0.97

* Abbreviations in this and later tables: A, adenine; G, guanine; C, cytosine; U, uracil; Pu, purine; Py, pyrimidine; RNA, ribonucleic acid.

TABLE II. *Purine analysis of RNA af* Drosophila melanogaster *eggs of two genetic types.**

(Moles of base/100 moles of phosphorus.)

	Egg type									
	XXY^{sc8}					XX				
				Ratio of G/A					Ratio of G/A	
Prep. #	Total purine	A	G	Purine analysis	Iono-phoresis	Total purine	A	G	Purine analysis	Iono-phoresis
1	50.2	27.6	22.4	0.81	0.80	43.1	20.8	22.2	1.07	1.04
2	48.6	26.0	22.0	0.85	0.82	42.8	19.9	21.2	1.12	1.06
Mean	49.4	26.8	22.2	0.83	0.81	43.0	20.4	21.7	1.10	1.05

* See Table I for abbreviations; age of eggs as in Table I: 3 hours.

phorus whereas the XX type RNA had only 0.430 moles. The guanine to phosphorus ratio was identical in the two types of RNA and it is clearly the difference in the amount of adenine which accounts for the higher purine content of the XXY^{sc8} RNA. The results obtained by this method therefore

TABLE III. *Molar absorptivity of RNA as affected by hydrolysis.**

RNA preparation	XXY^{sc8}			XX		
	ε(P)**260			ε(P) 260		
	pH 7	pH 1	pH 11	pH 7	pH 1	pH 11
unhydrolyzed	9,450	9,270	8,335	7,605	7,550	7,290
NaCl extracted; KOH – hydrolyzed	12,200	11,600	10,800	10,350	10,200	10,420
standard nucleotides	11,210	11,050	10,420	10,950	10,750	10,250
hot 9 % $HClO_4$ – hydrolyzed		11,900			8,207	

* See Table I for abbreviations; RNA from 3 hour eggs.
** ε(P) is molar absorptivity based on one gram-atom of phosphorus per liter.

agree with those obtained by ionophoresis of the alkaline hydrolysate. The guanine to adenine ratios are a further demonstration of the good agreement in the results obtained by the two methods.

To test whether the difference in adenylic acid content could be due to a low molecular weight, adenylic acid rich contaminant, a single experiment was performed on a sample of $X\overline{XY}^{sc8}$ RNA to investigate whether dialysis had any effect on the base composition. A sample of RNA was dialyzed in a rotating cellophane sac against a large volume of distilled water at 4°C for 36 hours. After concentration, the external phase was found to contain nucleotide material as judged by the presence of phosphorus and a typical nucleic acid shaped ultraviolet absorption curve. But of greater interest was the fact that the undialyzable material did not differ materially in base composition from the original undialyzed RNA, thereby indicating that the higher proportion of adenine in the XXY^{sc8} RNA was unlikely to be due to an adenine containing contaminant.

Ultraviolet absorption spectra.—These results allow a comparison of the ultraviolet absorption curves of the two types of nucleic acid in terms of the expectation from their nucleotide composition. It is well known [4] that the magnitudes of the extinction values of an RNA preparation are considerably influenced by the manner in which it is prepared and its subsequent treatment. We may first consider the comparison in Table III of the molar absorptivities, ε(P)'s [4], of the non-hydrolyzed salt extracted RNA, with he alkaline hydrolysate of the same preparation; and with that expected on the basis of the nucleotide composition of these preparations as calculated from the ε(P) values for standard nucleotides which were obtained from the graphs of Beaven, Holiday and Johnson [4].

On hydrolysis, the $\varepsilon(P)$ of a nucleic acid preparation increases considerably [4]. In Table III, a comparison can be made of the $\varepsilon(P)$'s of preparations of RNA extracted by hot salt with the $\varepsilon(P)$'s of these preparations: (1) degraded to mononucleotides by alkaline hydrolysis; and (2) treated with hot 9 per cent perchloric acid [27].

It is evident for both types of RNA, that the $\varepsilon(P)$ of the sodium chloride extracted preparations is increased after the perchloric acid hydrolysis. The extent of the increase is less for the XX RNA than it is for the $\overline{XX}Y^{sc8}$. Following complete alkaline hydrolysis to the mononucleotides, the $\varepsilon(P)$ values also increase in both types. However, the increase in $\varepsilon(P)$ following alkaline hydrolysis of the XX RNA is considerably greater than that following the acid treatment. In this respect there is a marked difference between the two types of RNA. Presumably, this difference is a reflection of the higher proportion of the purine linkages in the $\overline{XX}Y^{sc8}$ RNA which would be sensitive to the acid [18].

Among the mononucleotides, adenylic acid is distinguished by its high molar extinction value. Calculations of the $\varepsilon(P)$ value to be expected from a mixture of mononucleotides corresponding to the analyses of the base constitution of the two types of RNA are shown in the third line of Table III. The average difference between the calculated and experimental $\varepsilon(P)$ values at each of the three pH's is under 5 per cent. A more complete picture of the situation is shown in Fig. 2, which gives the complete absorption data for the three groups at the three pH's for each of the genetic types. The general shape of the experimental curves is indicative of clean nucleic acid preparations with relatively little protein contamination. It will be seen in the graphs that despite the concordance of the $\varepsilon(P)$ values at 260 mμ, the calculated mononucleotide curves are not superimposable on those of the alkaline hydrolysates of the nucleic acid preparations. These divergencies must be considered as a reflection of the influence of minor factors. It seems likely that these minor factors are concerned with the hydrolysis procedure, since although the RNA preparations were all the same, the hydrolysates on which the absorption spectra were taken were not identical with those used for ionophoresis. The shape of the curve is determined by the summation of the different nucleotides and the errors in their determination are independent; it is not surprising therefore that these minor disagreements occur.

We conclude that the ultraviolet absorption data confirm the analytical data in showing a difference in the constitution of the RNA in these two genetic types.

Amounts of RNA in the two types of eggs.—The RNA content of the unfertilized *Drosophila* egg has not previously been determined. It seemed advisable therefore not only to use the large frozen batches of eggs but also to make determinations on smaller numbers of freshly laid eggs. The characteristic RNA for each of the types was used as a standard, a point of some consequence considering the difference in base composition between them. The standard RNA was subjected to exactly the same treatment as the sample; the amount of RNA in it was determined from its total phosphorus content on the assumption that 9.5 per cent of its weight is phosphorus. As has already been noted, the DNA content of these samples is so low that it can be neglected in the present analyses. The problem of the DNA content of the egg will be treated in a separate communication.

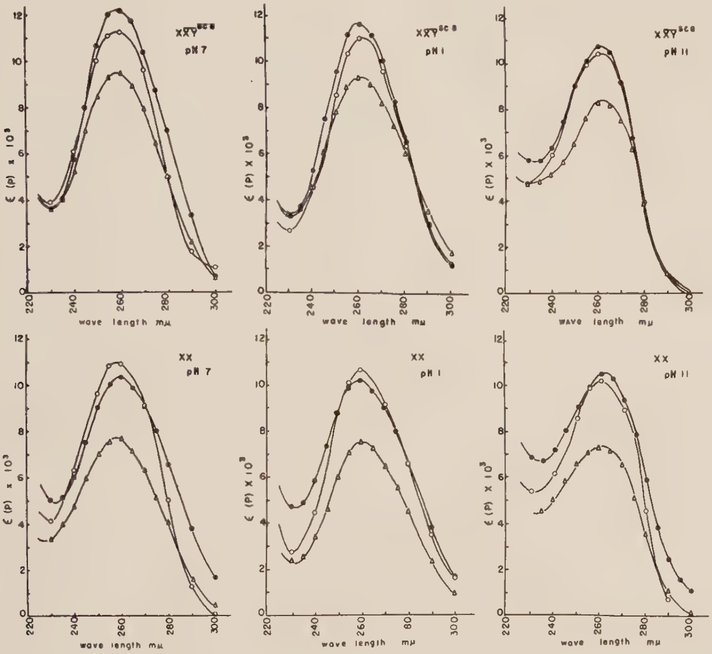

Fig. 2.—Ultraviolet absorption curves at three pH's of the purified RNA's of *D. melanogaster* eggs of two genetic types before and after alkaline hydrolysis as compared with the curves expected on the basis of the nucleotide composition of these RNA's. △——△ unhydrolyzed RNA; ○——○ KOH hydrolyzed RNA; ●——● standard nucleotides. The ordinates should read $\varepsilon (P) \times 10^{-3}$.

The nucleic acid content of the two types of *Drosophila* eggs as measured by ultraviolet absorption, phosphorus and orcinol analyses are shown in Table IV. On consideration of the $X\bar{X}Y^{sc8}$ group, the two sets of data show fair agreement; the average value for the three methods is 0.182 μg/egg for

Table IV. *Amounts of RNA in* Drosophila melanogaster *eggs of two genetic types.*

Micrograms per egg.

Collection	Sample #	Method of analysis: ultraviolet $X\overline{XY}^{sc8}$	ultraviolet XX	phosphorus $X\overline{XY}^{sc8}$	phosphorus XX	orcinol $XX\overline{Y}^{sc8}$	orcinol XX
125 eggs	1	0.188	0.206	0.185	0.191	0.179	0.182
	2	0.191	0.196	0.180	0.178	0.177	0.174
	3	0.192	0.213	0.173	0.197	0.177	0.199
	Mean	0.190	0.205	0.179	0.189	0.178	0.185
small batch	1	0.160	0.211	0.172	0.206	0.205	0.196
	2	0.164	0.216	0.167	0.168	0.192	0.195
	Mean	0.162	0.214	0.170	0.187	0.199	0.196

the 125 eggs as compared with 0.177 μg/egg for the small batches of frozen eggs. For the XX eggs, similarly, the values are 0.193 μg/egg for the 125 and 0.199 μg/egg for the small batches. In both series, it would appear that the amount of RNA is slightly higher in the XX eggs; the significance of this result is questionable. For the present, we interpret the results as indicating substantially the same amounts of RNA in the two types of egg.

Constitution of the RNA's in "24 hour" eggs.—The previous sections have described the base composition and RNA content of eggs collected over a three-hour egg-laying period. During this time the cytoplasm of the egg maintains its structure as examined by dark field microscopy (Fig. 1). On standing, a series of degenerative changes occurs, and the possibility therefore exists that such changes have already commenced during the period of egg collections. The difference between the two types of eggs might in some way be a consequence of differential rates of these degenerative changes. Accordingly, determinations were made on samples of RNA from unfertilized eggs allowed to remain on the medium for twenty-four hours before being collected.

The determination of the base composition of the RNA's from the two types of "24-hour" eggs are given in Table V. It is striking that the base composition now shows no important difference between the two types of egg. It does not seem likely therefore that degenerative changes can be responsible

TABLE V. *Purine and pyrimidine base distribution in RNA from two genetic types of* Drosophila melanogaster *eggs.**

(Moles of base/100 moles of phosphorus.)

Max. age (hours)	Prep. #	Sample #	Egg type									
			$X\overline{XY}^{sc8}$					XX				
			A	G	C	U	Pu/Py	A	G	C	U	Pu/Py
24	1	a	24.9	23.2	21.7	30.2		26.2	24.6	21.1	28.3	
		b	25.5	23.0	21.0	30.5		25.7	25.9	20.8	27.8	
		Mean	25.2	23.1	21.4	30.4	0.94	26.0	25.2	21.0	28.1	1.04

* See Table I for abbreviations.

TABLE VI. *Molar absorptivity of RNA as affected by hydrolysis.**

Max. age (hours)	RNA preparation	$X\overline{XY}^{sc8}$			XX		
		ε(P) 260			ε(P) 260		
		pH 7	pH 1	pH 11	pH 7	pH 1	pH 11
24	unhydrolyzed	7,500	7,550	7,150	8,900	8,700	9,000
	NaCl extracted; KOH – hydrolyzed	11,750	12,000	10,810	12,550	12,700	10,750
	standard nucleotides	10,950	10,650	10,000	11,000	10,750	10,350

* See Table III for abbreviations and explanations.

for the observed differences in the RNA base constitution of the three-hour eggs.

Obviously, the nature of the nucleic acids in these eggs which have been allowed to stand for twenty-four hours cannot be assumed to be identical with the nucleic acids of the freshly laid egg. Some indication of an actual lifference is given by the striking rise in the ε(P) of the alkaline hydrolysate (Table VI), particularly of the $X\overline{XY}^{sc8}$ group. Here the ε(P) of the unhydro-yzed sample is even lower than that of the XX group—a significant contrast o the situation in the three-hour eggs. Also, in both the XX and the $X\overline{XY}^{sc8}$ ypes, the alkaline hydrolysate shows a higher ε(P) than that calculated on the basis of the standard nucleotides. What enzymatic processes are responsible for these transformations cannot at present be stated. A possible key may lie in the analyses of the amounts of RNA per egg; incomplete data indicate that there is no striking decrease in the amount. In fact, the possibility of RNA synthesis is not excluded. It may be noted that significant changes (to be described in a succeeding communication) also occur in the ribosides and other nucleic acid components extracted by cold perchloric acid from the eggs.

DISCUSSION

The composition of the RNA's of a great many different animal and plant sources has been studied [24]. Differences in constitution have been found although the information at present is not sufficiently detailed to allow significant correlations between RNA structure and the specific biological properties of the source of the preparation. So far as we are aware, the present data give the only detailed analysis of insect RNA by modern methods. The base analyses are rather different from those of mammalian RNA or even from those of echinoderm egg RNA [13], or the early analysis of RNA from the blow-fly larva [14]. It is odd that the closest comparable base ratios are to be found among the plant viruses in which the guanylic and cytidylic acid fractions are also lower than the adenylic and uridylic acid fractions.

The distinctive point in the *Drosophila* analysis here presented is the effect of a genetic factor on the constitution of RNA in a specific cell type, the egg. It is not inconceivable that some of the variability noted in the base compositions of RNA from mammalian sources may be due to similar factors.

The question may be raised whether the observed differences in base composition between the two genetic egg types might actually be due to some artifact resulting from the experimental procedures employed. The identity of the isolated nucleotides can hardly be questioned, and it will be recalled that the amount of contaminating DNA was insufficient to influence the present analyses. Moreover, evidence has also been presented for the substantial purity of the isolated RNA preparations.

The possibility of a differential extraction of non-homogeneous, more or less tightly bound RNA species from one or other of the egg types must now be considered. A single preparation of XXY^{sc8} type RNA, extracted for only 2 hours instead of the routine 8 hours, had a significantly different base analysis from that reported in this paper; however, after exhaustive sodium chloride treatment, hot 9 per cent perchloric acid extracted only traces of protein-contaminated RNA, indicating that the major portion of the RNA had been removed by the sodium chloride. Hence, it appears unlikely that any differential extraction might have occurred.

For the RNA base analyses, it has been assumed that under the described conditions alkaline hydrolysis results in a quantitative yield of the constituent nucleotides, whereas acid hydrolysis leads to a complete liberation of the purine and pyrimidine bases. Both of these assumptions have recently been questioned [36, 32, 31). In the present experiments no evidence has been obtained that the expected hydrolysis products were not in fact precisely those to be anticipated. However, extraneous compounds, constituting not more than *ca.* 5 per cent of the total ultraviolet absorbing material employed for an analysis, would certainly have escaped detection.

The further question arises whether the differences in the base compositions between the two genetic types are really due to the synthesis of different types of RNA or to a differential breakdown of an originally identical RNA. A differential breakdown presumably could result from genetically different activities of some such enzymes as ribonucleotidases or phosphatases. The results obtained from allowing such putative enzyme action to take place in the unfertilized eggs during aging runs counter to this possibility; for it will be recalled that in the aged eggs the nucleic acids from the two genetic types approach each other in constitution.

The genetic (and biological) interest of the effect of the Y chromosome on the constitution of the RNA lies in its relevance to the hypothesis of the function of the heterochromatic chromosome regions of which the Y is a prime example. These regions have been postulated to act as controlling factors in the nucleic acid metabolism of the cell, and obviously the present data lend support to this hypothesis.

The original indication of the role of the Y chromosome in the synthesis of RNA came from the analysis of ultraviolet absorption data on the maturing oocytes [34]. At that time, the simplest interpretation of the data was that the amounts of RNA per cell were higher in the XXY than in the XX oocyte. The present data show clearly that the amounts in the matured egg are substantially the same; there may even be a suspicion of a decrease in the amount of RNA in the $\overline{XX}Y^{sc8}$ egg. It is of interest to note also that the presence or absence of the Y is without effect on the amount of RNA in either the isolated *Drosophila* salivary gland [30] or even in the adult insect [3].

The apparent contradiction between the cytophotometric data and the chemical analysis is resolved by consideration of the $\varepsilon(P)$ values. The higher $\varepsilon(P)$ of the $\overline{XX}Y^{sc8}$ RNA is sufficient to account for the difference of 15 per cent in the ultraviolet absorption of the two types of oocytes [34]. This is obviously a special case of the general proposition that for the estimation of RNA content it is essential to employ a standard of the same composition as the unknown.

The relation between the RNA in the oocyte and mature egg still remains to complicate the issue, and further study is still required of the course of RNA synthesis in the two types of egg. For the nucleic acids as extracted are a congeries of molecules and it is quite conceivable that different ones accumulate at different stages during the formation of the egg.

It should be emphasized that the effect of the Y chromosome on the nucleic acid metabolism of the egg is not limited to an effect on the RNA constitution. The analysis of the cold acid fraction to be described in succeeding communications has shown effects on nucleic acid precursors that are equally striking.

SUMMARY

1. To evaluate the relation of the heterochromatic chromosome regions to the nucleic acids, a comparison has been made of the nature and the amount of the ribonucleic acids in two types of unfertilized *Drosophila melanogaster* eggs; one, the normal wild type (XX), and the other containing the Y chromosome attached to one of the X's ($X\overline{XY}^{sc8}$).

2. The proportions of the purine and pyrimidine bases present in purified ribonucleic acids from both types of egg has been determined.

3. The XXY^{sc8} type RNA contains a significantly higher proportion of adenine than does the XX RNA.

4. The ultraviolet absorption curves of hydrolysates of the two types of RNA correspond to those expected for a mixture of nucleotides calculated from the analysis of the respective base compositions. Both before and after hydrolysis, the $X\overline{XY}^{sc8}$ RNA has a higher specific absorption than does the XX, in conformity with its higher content of adenine.

5. The quantity of RNA per egg is substantially equivalent in the XX and the XXY^{sc8} types.

6. In the light of the new chemical data, earlier measurements of the effect of the Y chromosome on the cytoplasm of the egg are now interpreted as indicating an effect of the Y on the composition instead of the amount of RNA in the egg cytoplasm. In this way, the hypothesis of a relation between the heterochromatic regions and the nucleic acid metabolism of the cell receives new support.

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II. NUCLEOSIDES AND RELATED COMPOUNDS IN THE ACID SOLUBLE FRACTION OF THE UNFERTILIZED EGG[1]

E. C. TRAVAGLINI, L. LEVENBOOK[2] and J. SCHULTZ

In the previous paper [16] of this series it has been shown that the base composition of the RNA in the unfertilized *Drosophila* egg is altered by the addition of a heterochromatic Y chromosome to the normal chromosome complement. It will be recalled that the heterochromatic character of the Y chromosome is evidenced by the high content of DNA per number of mutants on the map of metaphase chromosomes, and that heterochromatic chromosome regions have been postulated as controlling nucleic acid synthesis ([25]; for a review of earlier literature see [9]).

In a cell in which nucleic acid synthesis has proceeded rapidly and further activity is subject to a physiological block, as is the case in the unfertilized egg, an accumulation of possible nucleic acid precursor substances might reasonably be expected. Accordingly, we undertook a study to detect the presence of any such precursors in unfertilized XXY^{sc8} and XX *Drosophila* eggs and, as has already been briefly reported [14, 15] a wide variety of possible precursors was indeed found.

The nucleic acid precursor substances forming the subject of this paper consist of purine and pyrimidine bases and their nucleosides. There are numerous examples of such compounds occurring as normal cell constituents, but it is only quite recently that their diversity in a variety of cells and tissues has been realized; incorporation experiments employing suitable isotopes, have emphasized also the importance of these precursors in nucleic acid synthesis (for recent reviews see [12, 20, 3]; more general background can be found in [1, 22, 6]). It will be shown that there are indeed differences in the relative amounts of the bases and their nucleosides in the two genetic types.

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[1] Aided by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; by a grant from the U.S. Public Health Service C1613; and by an institutional grant of the American Cancer Society.

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EXPERIMENTAL

General.—A detailed description of the types of eggs, their collection, and preparation of the cold 1 per cent perchloric acid extracts forming the source material for the present analyses, has already been presented [16]. The ultraviolet absorption curves and total phosphorus, ribose and deoxyribose were determined on all extracts by the methods previously described; but for the more detailed analyses of the types and amounts of the various precursors, extracts from only the large egg batches (60,000–150,000 eggs) could yield sufficient material for the paper chromatographic procedures employed. Even these amounts were not sufficient for ion exchange chromatography, which would otherwise have been the method of choice.

It was early apparent (from preliminary experiments) that satisfactory resolution of the egg extracts employing either single or two-dimensional paper chromatography was complicated by a variety of fluorescent pigments, similar to those previously described for *Drosophila* by Hadorn and Mitchell [7]. Accordingly, two approaches were adopted: (1) acid hydrolysis to determine the presence of purine and pyrimidine bases; (2) identification of the various compounds by repeated chromatography and elutions.

Bases in hydrolysates of cold acid extracts.—Measured volumes of extracts obtained from known numbers of eggs were lyophilized and taken up in 1.0 ml of distilled water. Aliquots of these aqueous solutions were taken to dryness at 80°C and hydrolyzed to the bases employing a minimal volume of 72 per cent perchloric acid at 100°C [19]. The resulting hydrolysates were divided into two equal portions, and each was applied directly to a 5 × 40 cm sheet of Whatman No. 1 filter paper and chromatographed in either butanol–1 per cent ammonia [18] or isopropanol–HCl [26] for 18–24 hours at room temperature. After drying the paper, the ultraviolet absorbing spots were outlined in the light of a G.E. 8 W sterilamp provided with a Corning No. 9863 filter, and the R_f's of the different bases compared with simultaneous runs of known standards.

As indicated by a typical run shown in Fig. 4, the spots resulting from the above procedure were frequently ill-defined. For quantitative estimation under these circumstances, the paper was cut into 2 cm strips from the origin to the boundary, and each of these eluted in 1.0 ml of distilled water overnight. Spectrophotometric blanks were obtained from control strips similarly tested. Absorption spectra of the eluates were then measured at pH's 7, 1 and 11 in the Beckman spectrophotometer for comparison with known standards.

It was found that the content of an individual base from duplicate hydrolysates of the same extract varied on the average by about 17 per cent, although occasional variations of up to 45 per cent occurred in small samples. These larger variations may possibly be due to adsorption of bases onto the excessive amounts of carbon produced by the hydrolysis procedure.

Purine and pyrimidine compounds in the cold acid extracts.—As previously indicated, the chromatographic analysis of the extracts proved a difficult problem aggravated by the fact that acidic solvents could not be employed, due to the lability of purine deoxyribosides in acid media. Fig. 1 shows a contact print taken in the ultraviolet of a band chromatogram of an aqueous extract run in butanol-1 per cent ammonia.

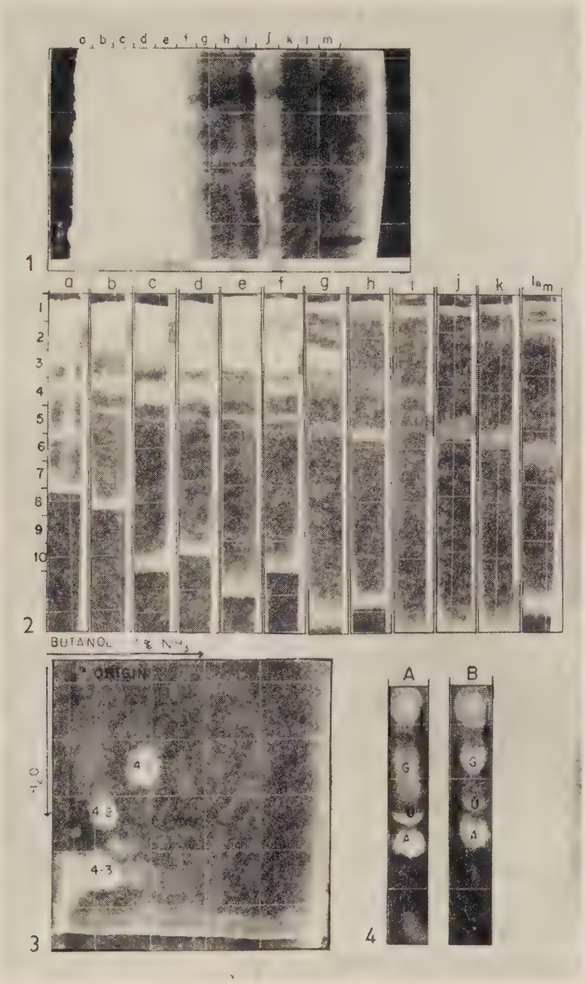
As shown by the light areas of ultraviolet absorption and/or fluorescence, the fluorescent pigments previously mentioned obscured and frequently prevented resolution of the compounds of interest. Attempts were made to analyze the compounds in the ultraviolet absorbing areas by cutting away the interfering pigments, elution, and subsequent re-chromatography. Although numerous compounds were identified in this manner, a different method was clearly necessary to reveal those substances possibly masked by the pigments.

For this purpose it was necessary to identify each region of the chromatogram according to its ultraviolet absorption and the presence or absence of pentose and phosphorus. Accordingly, after a band chromatogram of the extract was obtained and the paper dried, two longitudinal strips each $^1/_{20}$ the width of the chromatographic band were cut off (along a line perpendicular to the solvent front) and sprayed for deoxyribose [2] and phosphorus [8], respectively. A contact print such as that shown in Fig. 1 was made of the remaining portion of the chromatogram, and the paper was then cut into 1.3 cm strips (parallel to the solvent front) numbered consecutively from the origin to the solvent boundary. Each strip was eluted overnight in 2.0 ml of distilled water and 0.1 ml aliquots, diluted to 0.5 ml, were examined for ultraviolet absorption. This procedure served as a guide for further analysis, since it was found that, in general, samples showing significantly higher absorption at 260 mμ than 300 mμ corresponded eventually to discrete ultraviolet absorbing bands yielding, on further paper chromatography, purine and pyrimidine compounds. On the other hand, eluates with little or no 260 mμ absorption were never, in our experience, found to contain detectable nucleic acid components.

The remaining 1.9 ml of the selected eluates were concentrated for further chromatography. Distilled water made to pH 10 with *N* ammonium hydroxide was found to be useful as a chromatographic solvent [13]; not only did this rapidly separate several of the ultraviolet absorbing compounds, but in addition, the interfering pigments which had relatively low R_f's in alcoholic solvents, migrated close to the solvent front in water. Hence, by repeated chromatography with water, the pigments could largely be washed away.

The eluates, run in butanol–1 per cent ammonia, now revealed distinct ultraviolet absorbing bands (see Fig. 2). Tests for deoxyribose positive materials were performed on longitudinal strips 1 cm wide cut from each respective chromatogram. It will be observed that resolution of deoxyribose containing compounds has been considerably sharpened. The location of phosphorus containing compounds was not required at this step because they had been shown to be restricted to the origin band (band *a*) and the solvent boundary in the previous chromatogram.

For the final analysis, two dimensional chromatography was employed. The individual strip chromatograms were cut into subsections 2 cm wide and numbered from 1 to 10 from the origin to the solvent boundary (*cf.* Fig. 2). From each of the chromatograms similarly numbered segments were combined and eluted overnight, each eluate thus providing a pool of substances having closely similar R_f's. Aliquots of each eluate were hydrolyzed with 72 per cent perchloric acid and the 260 mμ absorption again determined; eluates whose hydrolysates did not show any significant absorption were discarded. Each selected eluate was then concentrated and chromatographed as a "spot" on Whatman No. 1 paper, 28 × 23 cm., first in water and subsequently in butanol–1 per cent ammonia (see Fig. 3).



Figs. 1–4.—Ultraviolet contact prints of a series of chromatograms resulting from the chromatographic analysis of a cold 1 per cent perchloric acid extract of unfertilized *Drosophila* eggs. The light areas denote the presence of ultraviolet absorbing and ultraviolet fluorescing compounds. The dotted areas next to chromatograms in Figs. 1 and 2 indicate the presence and relative concentration of deoxyribose containing compounds which have been located by spraying with cysteine-sulfuric acid.

Fig. 1.—Original band chromatogram (in butanol–1 per cent NH_3) of extract. Strips a, b,... etc. are cut parallel to solvent front.

Fig. 2.—Chromatograms (in butanol-1 per cent NH_3) of eluates from strips (a–m).

Fig. 3.—Two-dimensional spot chromatogram.

Fig. 4.—Duplicate chromatograms of extract hydrolysate, showing adenine (*A*), guanine (*G*) and uracil (*U*).

TABLE I. *Record of a typical chromatographic analysis of a cold 1 per cent perchloric acid extract of unfertilized* Drosophila *eggs.*[1]

(Fig. 2) Pool #	Hydrolysate products	2 dimensional chromatograms (Fig. 3)				Hydrolysate products	Sprays		Identity
		1	2	3	4		DR	P	
1	G, A	1.1	1.11	1.111		not tested			GR
				1.112		,,			AR
			1.12	1.121		,,			unstable in uv
				1.122		,,			,,
				1.123		,,			,,
				1.120	1.1201	,,			,,
					1.1202	,,			,,
					1.1203	,,			no 260 max.
					1.1204	,,		+	,,
					1.1205	,,		+	,,
			1.13	1.131		,,			
				1.132		,,			
2	G, H	2.1	2.11	2.111		G			GR
				2.112		H			HR
			2.12	2.121		H	+		HDR
			2.13	2.131		H			H
		2.2	2.21	2.211		G			GR
			2.22	2.221		H			HR
3	G, H, U	3.1	3.11						too dilute
			3.12	3.121		G	+		GDR
				3.122		H			HR
			3.13	3.131					too dilute
				3.132		U			UR
			3.14	3.141		H			HR
				3.142		U			UR
			3.15	3.151		G	+		GDR
				3.152		H			HR
				3.153		–			?
				3.154		U	+		UDR
		3.2	3.21			G			GR
			3.22	3.221		U			UR
4	U, A	4.1	4.11			–	+		no 260 max.
		4.2	4.21			U			UDR
			4.22			U+A			ADR
			4.23			too dilute			CR
		4.3				A			AR
5	0						+		

Table I (*continued*)

(Fig. 2) Pool #	Hydrolysate products	2 dimensional chromatograms (Fig. 3) 1	2	3	4	Hydrolysate products	Sprays DR	P	Identity
6	U, A, T	6.1	6.11			A	+		ADR
			6.12			too dilute	+		TDR
		6.2	6.21			A	+		ADR
		6.3	6.31			–			?
7–10	0								

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[1] Abbreviations: A, adenine; C, cytosine; G, guanine; H, hypoxanthine; T, thymine; U, uracil; AR, adenosine; CR, cytidine; GR, guanosine; HR, inosine; UR, uridine; ADR, deoxyadenosine; CDR, deoxycytidine; GDR, deoxyguanosine; HDR, deoxyinosine; TDR, thymidine; UDR, deoxyuridine; DR, deoxyribose; P, phosphorus.

Numbers: See text for explanation of numbers.

In some instances the "spots" obtained by the above procedure were compact and discrete, but more frequently they were clearly non-homogeneous. In either case the ultraviolet absorbing areas were cut out and eluted, the discrete "spots" to be identified by their R_f's, absorption spectra and pentose component, the non-homogeneous ones to be re-run on a second two-dimensional chromatogram. The two-dimensional chromatography was repeated up to four times, providing the maximum resolution obtainable using this particular procedure.

To distinguish further between deoxyribosides and ribosides, two methods were used: (1) purine deoxyribosides (when their concentration permitted) were identified by the diphenylamine reaction; (2) pyrimidine nucleosides were distinguished from each other by the method of Fox and Shugar [4] which involves determining the ultraviolet spectrophotometric behaviour of the nucleoside at pH's above 12.

Consideration of a typical experiment, shown in Table I, in conjunction with Figs. 1–3 will serve to illustrate how the identity of a particular spot could be related to the initial chromatogram of the egg extract.

The number "3" indicates the pool of substances having similar R_f's on strips a through m. In this experiment the purine and pyrimidine compounds were restricted to pools 1, 2, 3, 4 and 6. To maintain continuity during the succeeding two-dimensional chromatograms the following general convention was adopted: The pools of substances having closely similar R_f's (Fig. 2) were numbered from 1 to 10 followed by a decimal point. Each digit after the decimal point represented a subsequent two-dimensional chromatogram. The digit took its number from the position of spots in a series from the origin in the direction of the water solvent front. Thus deoxyuridine, for example, was obtained from spot 3.154; this indicates it was the fourth spot on the third two-dimensional chromatogram; this was derived from the fifth spot of the second chromatogram, which in turn derived from spot number one of the first two-dimensional chromatogram.

Source of reference compounds.—In the earlier stages of this work all reference compounds excepting the deoxyribosides were obtained from Schwarz Laboratories, Inc. (New York, N.Y.). Pure deoxyuridine was obtained through the kindness of Dr. W. S. McNutt; the other deoxyribonucleosides were prepared from a sample of purified deoxyribonucleic acid (kindly donated by Dr. Gerhardt Schmidt), by hydrolysis with deoxyribonuclease (Worthington Biochemical Labs., Freehold, N.J.), and purified intestinal alkaline phosphatase. Deoxyinosine was obtained by deamination of deoxyadenosine. As they became available, deoxyribosides from the California Foundation for Biochemical Research (Los Angeles, Calif.) were also employed.

RESULTS

Ultraviolet absorption spectra.—The ultraviolet absorption curves at pH's 7, 1 and 11 were routinely determined for each of the cold acid extracts. Fig. 5 shows representative curves for the XX and $X\overline{X}Y^{sc8}$ eggs. It is evident that the curves for both extracts have maxima and minima characteristics of purine and/or pyrimidine containing substances. Hadorn and Mitchell [7] have shown that the fluorescent substances in *Drosophila* eggs contain tryptophan derivatives and pterins, the presence of either of which would contribute considerably to the absorption of these extracts. Hence, only gross

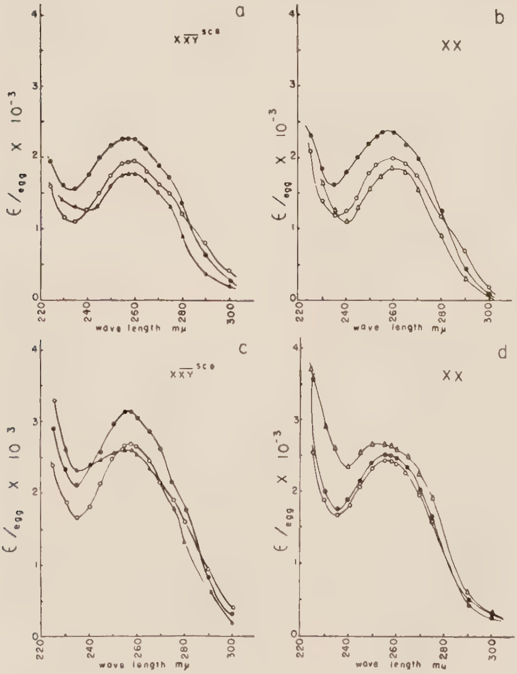

Fig. 5.—Comparison of the ultraviolet absorption spectra at pH's 7, 1, and 11 of cold 1 per cent perchloric acid extracts of 3-hour (*a* and *b*) and "24"-hour (*c* and *d*) unfertilized *Drosophila* eggs of two genetic types, $X\overline{X}Y^{sc8}$ and XX.
Symbols: ●——● pH 7; ○——○ pH 1; △——△ pH 11. The ordinates should read ε/egg × 10^3.

TABLE II. *Acid soluble phosphorus, ribose and deoxyribose in XX and* $X\bar{X}Y^{sc8}$ *eggs of* D. melanogaster.

				ortho P		total P		ribose		deoxyribose	
				Micromoles × 10^{-6}/egg							
	Age	Eggs	Sample #	$X\bar{X}Y^{sc8}$	XX	$X\bar{X}Y^{sc8}$	XX	$X\bar{X}Y^{sc8}$	XX	$X\bar{X}Y^{sc8}$	XX
A	½ hr.[1]	fresh	1			572	581	201	220	11	10
			2			621	545	215	209	9	10
			3			572	559	206	215	13	11
			Mean			590	560	208	215	11	11
	3 hr.[2]	frozen	1	134	144	531	504	192	154	4	7
			2	239	174	790	578	188	202	6	4
			3	207	295	504	545	209	191	7	7
			4		200		675		130		8
			Mean	193	203	608	575	197	169	6	7
B	24 hr.[3]	frozen	1	220		810		246		8	
			2	130		435		123		6	
			3	309		760		240		4	
			Mean	220		668		203		6	

[1] Samples of 125 eggs counted out directly after laying.

[2] Samples of 3000-4000 eggs collected after 3-hr. period of egg laying and stored in the frozen state.

[3] Samples of 3000–4000 eggs collected after a 24-hr. period of egg laying (minimum age 12 hrs.) and stored in the frozen state.

differences between the two genetic types may be detected from these ultraviolet absorption spectra, and actually no consistent variation was found from examination of 21 extracts of each egg type. However, more detailed examination of the individual extracts has shown that differences do exist in many of the ultraviolet absorbing components.

Comparisons of phosphorus, ribose, and deoxyribose analyses.—Possible gross differences between the cold acid extracts of the two types of eggs were also examined by analyzing the phosphorus, ribose and deoxyribose content. These data are presented in Table II*A*, from which it is evident that there is no significant differences between the two genetic types of "3-hour" eggs. Further, there was no major difference in the phosphorus content of either the alcohol ether extracts (mean value 251×10^{-6} μmoles/

TABLE III. *Base composition of 72 per cent perchloric acid hydrolysates of cold 1 per cent perchloric acid extracts of XX and* $X\overline{X}Y^{sc8}$ *eggs of* D. melanogaster.[1]

	Egg type	Collection	Hyd. #	micromoles × 10^{-6}/egg					Total bases	Total Pu	Total Py	Pu/Py
				A	G	C	T	U				
A	$X\overline{X}Y^{sc8}$	a	1	33.7	15.4	—	1.00	16.0				
			2	33.7	17.8	—	1.46	19.8				
			Mean	33.7	16.6	—	1.23	17.9	69.4	50.3	19.13	2.6
	XX	a	1	10.2	8.6	3.9		11.2				
			2	7.9	4.9	5.8	—	11.5				
			Mean	9.1	6.8	4.9	—	11.4	32.2	15.9	16.3	1.0
		b	1	9.3	7.6	4.6	—	11.5	33.0	16.9	16.1	1.1
B	XXY^{sc8}	a	1	22.1	24.2	—	0.97	18.6				
			2	19.2	28.9	—	2.64	18.7				
			Mean	20.7	26.6	—	1.80	18.7	67.8	47.3	20.5	2.3

[1] Abbreviations: A, adenine; G, guanine; C, cytosine; T, thymine; U, uracil; Pu, purine; Py, pyrimidine.

egg), or of the hot 9 per cent perchloric acid extract [16]; no residual phosphorus could be detected in the eggs. The total phosphorus content of the XXY^{sc8} was 1.37×10^{-3} μmoles/egg and XX, 1.42×10^{-3} μmoles/egg. (King found the total phosphorus content to be 1.64×10^{-3} μmoles/egg [11]). The total phosphorus in each type egg was accounted for to the extent of 92 per cent.

The presence of large amounts of deoxyribose in both egg types is of particular interest. More extensive data on the deoxyribose content and a detailed discussion thereof will be presented in a subsequent paper.

Since gross ultraviolet spectra and chemical measurements of the cold acid extracts of the XX and XXY^{sc8} eggs showed no essential dissimilarity, attention was next focussed on the base analysis of the corresponding acid hydrolysates.

Hydrolysates.—The results of chromatographic analyses of the 72 per cent perchloric acid hydrolysates of the cold acid extracts are shown in Table III*A*. All the purine and pyrimidine bases found in either the ribo- or deoxyribo-nucleic acids, with the exceptions of 5-methyl and 5-hydroxymethyl cytosine, appear in the hydrolysates of one or the other of the two egg types. Probably due to the unsatisfactory resolving properties of the particular solvents employed, no hypoxanthine could be detected in the hydrolysates, but it will be shown below that hypoxanthine derivatives are present in the unhydrolyzed extracts.

Comparison of the two genetic types reveals differences in the proportions of purines in the hydrolysates, the adenine content of the $XX\bar{Y}^{sc8}$ eggs being about for times and the guanine content about twice that present in the XX egg. Although the adenine and guanine concentrations presented in Table III may include some hypoxanthine, nevertheless, the ratio of the total purines of the $XX\bar{Y}^{sc8}$ eggs to those of the XX type is about 3:1. Since the pyrimidine content of each egg type is approximately the same, the ratio of total $XX\bar{Y}^{sc8}$ purines to pyrimidines is more than double that for the XX eggs.

The differences amongst the pyrimidines of the two egg types are practically qualitative; cytosine is not present in the $XX\bar{Y}^{sc8}$ type in detectable amounts, whereas it constitutes about one-third of the total pyrimidines of the XX eggs. Conversely, thymine occurs regularly in the $XX\bar{Y}^{sc8}$ hydrolysates but is not found in those of the XX eggs.

Analysis of compounds initially present in the cold acid extracts.—In order properly to evaluate the analyses of the free purine and pyrimidine containing compounds in the *Drosophila* eggs, consideration should be given first to the reliability of the measurement and identification of these materials by the procedures employed. As many as six successive chromatograms were required before some particular spots could be identified. Such a procedure is attended by inevitable losses. In the extreme case this may result in the complete "disappearance" of some components, but more generally results in lower recoveries of the compounds being estimated. For example, in the particular experiment summarized in Table I, the determination of the total amount of guanosine (which occurred in spots 1.111, 2.111, 2.211 and 3.21) depended upon 15 separate elutions. On the other hand, better recoveries of most of the pyrimidines and some purines were obtained as a result of their initially sharper separation on the strip chromatograms. Thus, deoxyguanosine, for example, occurred only in spots 3.121 and 3.151, and could therefore be estimated with fewer elutions.

Table IV*A* lists the identified compounds estimated on a per egg basis. It will be seen that of the 14 compounds identified, most of the nucleic acid precursors occur as ribosides and deoxyribosides, while the free bases hypoxanthine, adenine and uracil are only minor components. The complete absence of guanine is particularly striking. It may be noted that although no nucleotides were found, this does not necessarily mean that these were absent from the extracts; phosphorus, possibly nucleotide phosphorus, was normally found in the pigmented region near the origin of the initial chro-

Table IV. *Purine and pyrimidine compounds found in the cold 1 per cent perchloric acid extract of* $X\overline{X}Y^{sc8}$ *and XX eggs of* D. melanogaster.[1]

				Micromoles × 10⁻⁶/egg																
				Purines									Pyrimidines							
Experiment	Egg type	Sample No.	No. of eggs × 10³	A	AR	ADR	G	GR	GDR	H	HR	HDR	C	CR	CDR	T	TDR	U	UR	UDR
A	$X\overline{X}Y^{sc8}$	1	241	—[2]	1.2	—	—	1.4	0.5	0.5	3.0	0.2	—	—	—	—	0.8	1.8	3.1	0.4
		2	297	—	1.1	0.1	—	0.6	0.4	0.5	2.2	0.5	—	—	—	—	0.2	—	1.5	0.5
		Mean	269	—	1.1	0.1	—	1.0	0.5	0.5	2.6	0.3	—	—	—	—	0.5	0.9	2.3	0.4
	XX	1	129	0.1	0.2	—	—	0.2	0.3	0.4	0.3	—	—	0.2	—	—	0.2	1.6	2.0	0.8
B	$X\overline{X}Y^{sc8}$	1	97	0.2	1.0	0.7	0.7	0.3	0.3	—	1.0	—	0.1	—	—	—	0.7	—	2.9	0.5

Abbreviations: see Table I.

[1] The previous summary of these results (25) contained several errors as follows: the amounts given above as micromoles × 10^{-6}/egg were expressed incorrectly as micromoles/egg; re-evaluations of the chromatograms have changed some of the numbers, particularly in the case of deoxyguanosine in the aged eggs (B above).

[2] The symbol (–) indicates no perceptible amount.

TABLE V. *The total amounts of purines and pyrimidines found in the cold acid extracts as compared with the amounts determined in the hydrolysates of these extracts, to assess the relative recovery in the former.*[1]

Egg Type	C			T			U			Total pyrimidines			Total purines		
	s	b	s/b	s	b	s/b	s	b	s/b	s	b	s/b	s	b	s/b
$X\overline{XY}^{sc8}$	—	—	—	0.5	1.2	40.6	3.6	17.9	20.0	4.1	19.1	21.5	6.1	50.3	12.1
XX	0.2	4.8	5.0	0.2	—	—	4.4	11.5	38.0	4.8	16.3	29.4	1.4	16.4	8.8
$X\overline{XY}^{sc8}$	0.1	—	—	0.7	1.8	36.0	3.4	18.7	18.2	4.1	20.5	20.1	3.4	47.3	7.3

[1] Abbreviations: s, amount found in spot analyses (micromoles $\times 10^{-6}$/egg); b, amount found in hydrolysate analyses (micromoles $\times 10^{-6}$/egg); s/b, percentage recovery.

matogram; during the subsequent chromatography, however, no ultraviolet absorbing compounds containing phosphorus could be isolated.

Deoxyribose unassociated with ultraviolet absorbing material was located in two regions on the strip chromatograms (pool 5 and 7 10, Table I). One of these (pool 5) agreed in R_f with free deoxyribose; no attempt was made to identify the other.

In two experiments, unsuccessful attempts were made to identify several ultraviolet absorbing spots having $R_f > 0.7$ in butanol 1 per cent ammonia, and unstable to 72 per cent perchloric acid hydrolysis. Several of these had double peaked absorption spectra with maxima around 235 ± 5 and 290 ± 10 mμ, respectively; but none agreed with known compounds in all details.

Inspection of Table IV*A* reveals differences between XXY^{sc8} and XX eggs. As stated at the beginning of this section, the quantitative values in Table IV*A* entail variable losses. For a meaningful comparison between the two egg types, the magnitude of these losses was assessed on a per egg basis by expressing the sum of the molar concentrations of the compounds containing the same base in Table IV*A*, as a percentage of the amount of this base recovered in the corresponding hydrolysates (Table V*A*).

In view of the failure to identify free hypoxanthine in the hydrolysates, it was decided to sum the total purine for the purpose of estimating recoveries of the purine precursors. It will be seen in Table V*A* that purine compounds are recovered to the same extent in both egg types, viz., about 10 per cent. As expected, recoveries for the pyrimidine compounds were higher (about 25 per cent in each case) except for cytosine, the amounts of which were so small that low recoveries are not surprising. Since the percentage recoveries of the various compounds from the two egg types are similar, it seems probable that a comparison of the concentrations of purine and pyrimidine compounds in $\overline{XX}Y^{sc8}$ and XX eggs is valid.

The concentration of total purine compounds in $\overline{XX}Y^{sc8}$ eggs is approximately four times as high as in XX eggs—the same ratio as already found for the total purines in the corresponding hydrolysates.

Table IV*A* shows that free adenine is not found in $\overline{XX}Y^{sc8}$ eggs but is present in XX eggs, in approximately the same amount as adenosine. A difference is also seen in the ratio of inosine to hypoxanthine; in the XXY^{sc8} eggs this ratio is about 5, in the XX egg it is only about 0.70. The remaining pyrimidine compounds appear to be similar in the extracts of the two egg types.

The analyses of the pyrimidine compounds shown in Table IV*A* extend the results previously found for the hydrolysates. Only very small amounts of cytidine were found in XXY^{sc8} eggs, the concentration being about one-tenth of that in the XX eggs. No other cytosine compounds were detected. On the other hand, the XXY^{sc8} egg had a higher content of thymidine. Uracil, the major free base in both types of egg, was found in the single XX sample, and in 3 out of 5 extracts of XXY^{sc8} eggs. The deoxyuridine content of XX eggs appears to be about twice as high as that of the $\overline{XX}Y^{sc8}$ type.

Composition of cold acid extracts in aged eggs.—The possibility that the unfertilized eggs as collected had undergone degenerative changes has already been tested with respect to the base composition of the RNA's through a study of eggs aged for 24 hours [16].

Analysis of the cold acid extract of the "24-hour" aged eggs (minimum age: 12 hours after egg laying) was performed on XXY^{sc8} eggs only. A comparison of these data (Section B of Tables II, III, IV and V) with the corresponding values for the 3-hour eggs in Sections *A* of these Tables indicates that no significant changes have occurred in the content of phosphorus, ribose or deoxyribose. Although the total amount of purines and pyrimidines in the perchloric acid hydrolysate is similar to that of the 3-hour eggs, there does appear to be some indication of an increase in the amount of guanine and thymine, but a decrease in the adenine content.

The size of the aged egg collection was barely adequate for the one analysis of free bases, ribo- and deoxyribosides, and hence the apparent quantitative differences between the aged and 3-hour eggs shown in sections A and B, Table III, must be evaluated with caution. The following points of comparison, however, are noteworthy: Some free adenine and considerable quantities of free guanine—neither of which were detected in the 3-hour egg—were

TABLE VI. *Comparison of individual bases, expressed as molar percentages, in the acid soluble pool and in the RNA of* D. melanogaster *eggs of 2 genetic types:* $XX\overline{Y}^{sc8}$ *and XX.*

	Adenine		Guanine		Cytosine		Uracil	
Source	$XX\overline{Y}^{sc8}$	XX	$XX\overline{Y}^{sc8}$	XX	$XX\overline{Y}^{sc8}$	XX	$XX\overline{Y}^{sc8}$	XX
72 % $HClO_4$ hydrolysate cold acid extract	48.5	28.0	24.0	23.0		13.9	28.5	34.8
RNA	29.9	25.0	23.4	23.2	18.5	20.4	28.5	30.3

found in the aged $XX\overline{Y}^{sc8}$ egg. Conversely hypoxanthine is no longer detectable. It appears further that there is a decrease in the inosine, and an increase in the uridine content of the 24-hour eggs. Considering the small size of the egg batch, the appearance of cytosine even in trace amounts is striking especially since no cytosine could be detected in the much larger samples of 3-hour $XX\overline{Y}^{sc8}$ eggs.

We may now consider to what extent the precursor substances of the aged eggs can be indicative of possible early catabolic changes. If these substances were accumulating in the egg due to polynucleotide degradation, then it would be anticipated that the quantity of such fragments would be higher in the aged than in the 3-hour eggs. This is not, however, the case; the changes in the composition between the 3- and 24-hour egg extracts cannot easily be explained by a simple breakdown of either RNA or purine and/or pyrimidine compounds. Further, the degenerative processes in the $XX\overline{Y}^{sc8}$ 24-hour eggs do not cause the composition of these egg extracts to approximate that of the 3-hour XX eggs, particularly in the total purine content. The degenerative processes in the ageing eggs are clearly complex and further work will be required to follow quantitatively the interrelationships between the nucleic acid components.

DISCUSSION

The present findings have implications not only for the possible biochemical function of the heterochromatic Y chromosome, but also for the problems of nucleic acid synthesis in general. The substances found in the cold acid extracts here considered must be, from their very nature, either precursors or degradation products of the nucleic acids. While there is no direct evidence in *Drosophila* tracing the pattern of nucleic acid synthesis by the incorporation of isotopically labelled compounds, it is likely that the general outline of the schemes [12, 20, 3, 1, 22, 6] worked out for various microorganisms and vertebrate tissues will apply here also [25, 22a]. The compounds of the cold acid extract—purine and pyrimidine ribosides and deoxyribosides—are the substances of the so-called "salvage" [12] mechanism for nucleic acid synthesis, as contrasted to the *de novo* synthesis from compounds below the purine and pyrimidine level.

Among the fourteen compounds identified in the cold acid extract of the *Drosophila* egg, all but one can be related to RNA or DNA as either actual components or the deamination products thereof. The presence of deoxyuridine is of particular interest in view of its precursor relation to thymidine [5, 21]. Our tentative record of the occurrence of this substance in the *Drosophila* egg was the first, so far as we are aware, in animal tissues. Schneider has shown that the extent to which this compound accumulates may vary in different tissues, e.g., normal liver as compared with hepatoma in the rat [23]. His earlier conclusion of the occurrence of deoxyuridine in the normal liver he now interprets as an artifact, due to deamination of deoxycytidine during paper chromatography. This had also occurred to us, hence the tentative nature of our earlier report [14]. However, the solvents we used were found not to deaminate cytosine or its derivatives on paper (the solvent used in the rat work was butanol 5 per cent boric acid). Another possibility, that deamination occurred during the three-hour egg collection is likewise improbable, since the uracil content is scarcely increased in the aged eggs. It is therefore most likely that deoxyuridine is a normal constituent of the *Drosophila* egg.

In view of the variety of identified precursors, and the at least equal number of ultraviolet absorbing compounds as yet unidentified, the apparent absence of certain nucleic acid components, specifically all nucleotides and free guanine, is of interest. As already mentioned above, the interfering egg pigments and the many elutions with attendant losses during paper chromatography could easily obscure materials present in low concentration. Both guanine and the nucleotides would, however, readily have been identified and it seems most likely that they are only minor components of the extracts. The nucleotides, labile as they are known to be in tissue homogenates, could easily have been dephosphorylated to nucleosides during the period of egg collection. It is also not unlikely, as in the case of the deoxyriboside compounds of normal liver [24] and irradiated bacteria [10], that in the egg these accumulate before the stimulus to DNA synthesis at fertilization when the phosphorylation mechanism could be activated. In view of the importance of phosphorylated compounds in the *de novo* pathways of purine and pyrimidine synthesis (for an excellent review see [3]), the accumulation of the nucleosides in the *Drosophila* material necessitates further analysis for its understanding. With regard to the absence of free guanine, it should be noted that considerable quantities of its ribo- and deoxyribonucleosides are present in the extracts.

The evidence for an effect of the Y chromosome on the constitution of the egg RNA has already been presented [16]. We may now consider the relation of the putative precursors of the nucleic acids to the RNA's in the same types of egg, leaving the small amount of DNA out of consideration. Even though enzymatic synthesis of polynucleotides *in vitro* has not yet been accomplished with extracts from animal tissues [20], the background afforded by this work indicates that the composition of a given nucleic acid can be regarded as somehow a function of the composition of the pool of precursors available for synthesis, and of the nature of the enzymes responsible for the assembly of monomers into the polynucleotides. There is no information concerning the polynucleotide phosphorylases of *Drosophila*. However, there is a clear relation between the amounts of the different bases in the cold acid extracts and their proportions in the RNA of the two genetic types: The bases which are relatively high in a particular RNA are also present in high amounts relative to the others in the cold acid-soluble fraction. This appears in Table VI, where the percentage base compositions of the cold acid extracts and the corresponding RNA's are compared. In addition there are differences in the absolute amounts of the purines per egg; the total purine compounds synthesized are greater in the $XX\bar{Y}^{sc8}$ than in the XX egg, as already noted (cf. pp. 70 and 72, Tables III and IV). These facts imply that the further analysis of the effects of the Y chromosome requires insight into the synthesis of the bases; for if the pool of precursors were the same in both genetic types initially, it would be expected that the increase of a component in the RNA would entail its decrease in the acid soluble fraction. This is not the case: the differences in the composition of the RNA's reflect a much wider variation in the pool of precursors.

The existence of quantitative differences in both purines and pyrimidines suggests that the effect of the Y takes place at a level common to both types of base, possibly the transfer of single carbon units. To investigate these problems will require not only direct studies of the enzymatic content of the eggs, but also improved methods of estimating the purine and pyrimidine compounds. For the deoxyribosides, and for thymidine specifically, bacterimetric methods have proved useful; the results of Travaglini and Schultz, confirming and extending those obtained by the chromatographic methods of the present paper, have already been summarized [25]. They will be reported separately, and a more detailed discussion of the deoxyribosides is deferred on this account.

Considered as a case in the biochemical genetics of nucleic acid synthesis, the present analysis presents some points of difference from the usual investigation of metabolic pathways by the selection of mutants deficient for a particular step accomplished by the normal genotype. In the mutants (e.g., those studied in the microorganisms) the reaction blocked by the loss of function at the mutant locus leads to ramifying changes in the metabolic network. The changes in accumulation of precursors and in nucleic acid composition reported here come about from the addition of the synthetic capacities of the Y chromosome to the chromosome complement. Moreover, the heterochromatic regions, of which the Y chromosome contains one group, are represented elsewhere in the genome also (for a review see Hannah [9]). The homologies between the different heterochromatic regions are not precise, and they may turn out to contain pseudoalleles (compare Lewis [17]), having to do with different steps in nucleic acid formation. In any case, it is noteworthy that the addition of the heterochromatic Y has a considerable effect on the course of RNA synthesis, while the total amounts (see the previous paper) remain limited by other factors in the genotype.

SUMMARY

1. The purine and pyrimidine-containing compounds in the cold acid-soluble fraction of the unfertilized *Drosophila melanogaster* egg have been investigated, in order to compare the contents of these substances in the two genetic types (XX and XXY^{sc8}) in which differences in the RNA composition have already been found.

2. Identification of 14 compounds has been accomplished by determination of their R_f's on paper, ultraviolet absorption spectra at 3 pH's and by tests for deoxyribose, ribose and phosphorus. The quantities found were compared with analyses of similar extracts after hydrolysis to the free bases, to gain estimates of recoveries. Although low, these were the same for the extracts from both genetic types, permitting comparisons to be made.

3. No nucleotides were recovered from these extracts, the major components being ribosides and deoxyribosides, with free bases present to a lesser degree. All the usual purine and pyrimidine bases with the exception

of 5-methylcytosine have been found; in addition, some unidentified compounds having absorption maxima around 235 and 290 mμ in the ultraviolet were isolated chromatographically.

4. The presence of deoxyribosides, and particularly of deoxyuridine in the extracts, is noteworthy in relation to the synthesis of DNA.

5. The content of purine compounds in the extracts from $X\overline{XY}^{sc8}$ eggs is more than double that of the XX type; the chief difference is due to adenine containing compounds. Although the total content of pyrimidines is the same in the two types, the proportions differ: cytosine is present in only trace amounts in the $X\overline{XY}^{sc8}$ type, as compared with sizable amounts in the XX, the converse being the case for thymine.

6. The existence of effects of the Y chromosome on the pool size of purine compounds, on the proportions of the pyrimidines, as well as on the base composition of the RNA, may indicate the site of action of this chiefly heterochromatic chromosome to be on the synthesis of the nucleic acid bases.

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Experimental Cell Research **15**, *80–84* (*1958*)

INTRACELLULAR LOCALIZATION OF IRON, CALCIUM, MOLYBDENUM AND TUNGSTEN IN *AZOTOBACTER VINELANDII*[1]

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Received October 6, 1957

THE demonstration of protoplast formation by Weibull [10] has been followed by the development of techniques for cell rupture involving osmotic lysis of protoplasts or the direct lysis of whole cells by various reagents. These techniques are especially useful for recovering the membrane ghost fraction. Sonic oscillation or cell grinding is apparently too violent to allow recovery of this delicate structure.

We will report in this paper the localization of various metals in fractions of *Azotobacter vinelandii* strain O obtained by direct lysis of whole cells or protoplasts as described by Repaske [6, 7]. Optical and electron micrographs and dry weight measurements are presented as evidence of the nature of the fractions.

Localization of iron is of interest in relation to its role in the cytochrome system. Cytochromes have been reported to be components of the envelope of *Azotobacter* [8]. The recognized requirement of molybdenum in nitrogen fixation, nitrate reduction, and possibly in hydrogenase lends interest to the localization of this metal. Furthermore this strain of *Azotobacter vinelandii* (strain O) incorporates large excesses of molybdenum which may function in an alternate terminal oxidase [4, 5]. Tungsten has been reported to be a competitive inhibitor of molybdenum in *A. vinelandii* [5, 9] and might be expected to have a distribution similar to molybdenum. Finally calcium has been reported by Esposito and Wilson [2] to be specifically required for nitrogen fixation by this organism. Therefore the localization of calcium is of interest.

METHODS

Cells were grown on a modified, nitrogen-free Burk's [1] medium plus 3 per cent sucrose. Cultures were incubated with the labeled metals at the following levels: Mo^{99} as $Na_2Mo^{99}O_4$ at 4 ppm, Ca^{45} as $Ca^{45}Cl_2$ at 7 ppm, Fe^{59} as $Fe^{59}Cl_3$ at 0.1 ppm,

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[1] This research supported in part by a grant from the Research Corporation.

and W^{185} as $K_2W^{185}O_4$ at 40 ppm (W/Mo ratio of 40). The cells were harvested in the late log phase. At this point the cells were lysed or protoplasts formed by the methods of Repaske [6, 7]. Membrane ghosts and protoplasts were collected by centrifugation at 2000 × gravity. Radioactive assays were made with a Geiger-Müller end window tube and standard scaling circuits.

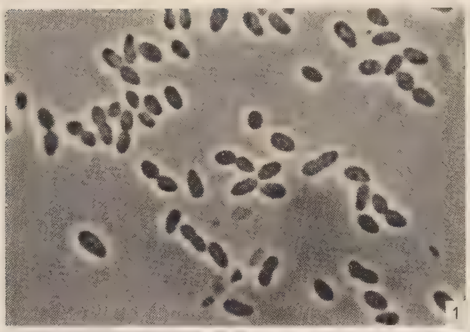
RESULTS AND DISCUSSION

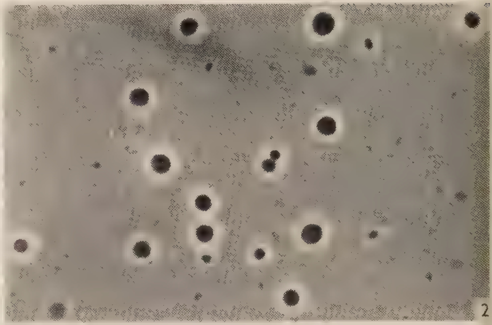
In Table I are reported results of experiments in which the localization of the above metals was determined in *Azotobacter* fractions obtained by direct lysing of whole cells by the methods of Repaske [6, 7]. Fraction 2 shows that of the total activity which remained after washing twice with distilled water (fraction 1), less than 25 per cent of the Mo^{99}, Ca^{45}, and W^{185} was found in the membrane fraction. When the membrane fraction was washed and resuspended in distilled water (fraction 4), most of the calcium, molybdenum and tungsten was lost. This indicates that molybdenum, tungsten and calcium are not associated with the membrane fraction, other than at very low levels. On the other hand $\frac{2}{3}$ of the total Fe^{59} incorporated was found in the membrane fraction. One-half of the total Fe^{59} remained in this fraction after washing.

A high count in fraction 3, the membrane supernatant fraction, does not distinguish original localization in the cell wall from an intracellular localization. Such information may be obtained by the technique of protoplast formation. When protoplasts are formed directly, the activity found in the supernatant fraction, after the protoplasts are sedimented by centrifugation, may be ascribed to the original cell wall material. This assumes that no protoplast rupture occurs (or that corrections can be made for such rupture), that the protoplasts have the same permeability characteristics as the original cells,

TABLE I. *Localization of various metals in* Azotobacter vinelandii *fractions obtained by direct lysis with lysozyme.*

Fraction	Mo^{99}	Ca^{45}	Fe^{59}	W^{185}
1. Twice washed resuspended cells	1525	2990	2650	130
2. Resuspended membrane ghosts	155	40	2042	25
3. Membrane supernatant	1200	2900	800	96
4. Washed membrane ghosts	30	0	1360	10
5. Wash losses of Fraction 4	125	10	370	20





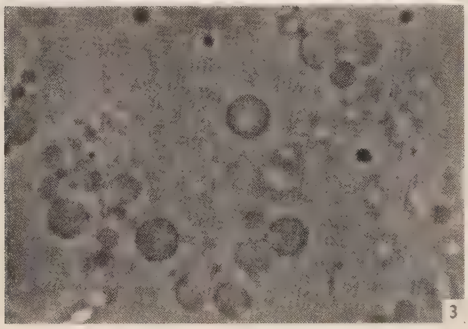


Fig. 1.—Log phase *Azotobacter vinelandii*. × 970.

Fig. 2.—Protoplasts. × 970.

Fig. 3.—Membranes. × 970.

and that the cell wall components are dissociated from the protoplast and resist sedimentation at 2000 times gravity.

Using Repaske's [6, 7] technique for preparing protoplasts (lysozyme and versene in tris buffer at pH 8 with 0.33 *M* NaCl), it was found that Mo^{99}, Fe^{59}, and W^{185} were present within the protoplasts. With subsequent osmotic rupture of the protoplasts the Fe^{59} was once again found largely in the membrane ghosts while the Mo^{99}, and W^{185} were localized in the supernatant fraction. These results indicate the absence of molybdenum, iron, or tungsten in cell wall material. With Ca^{45}, however, most of the label was found in the supernatant fraction after protoplast preparation. This may indicate that calcium is largely a component of the cell wall material, although experiments have shown that versene alone will remove a major portion of the Ca^{45} retained in washed whole cells.

The membrane fraction of *A. vinelandii* contains components other than the original membrane, some of which can be washed out [6, 7]. Assuming a thickness of 50 Å for the membrane, one would not likely find more than 10 per cent of the dry weight of the cell in the membrane. We have examined the dry weights of various fractions prepared by the above methods, and found that the membrane fraction obtained by direct lysozyme lysis contains

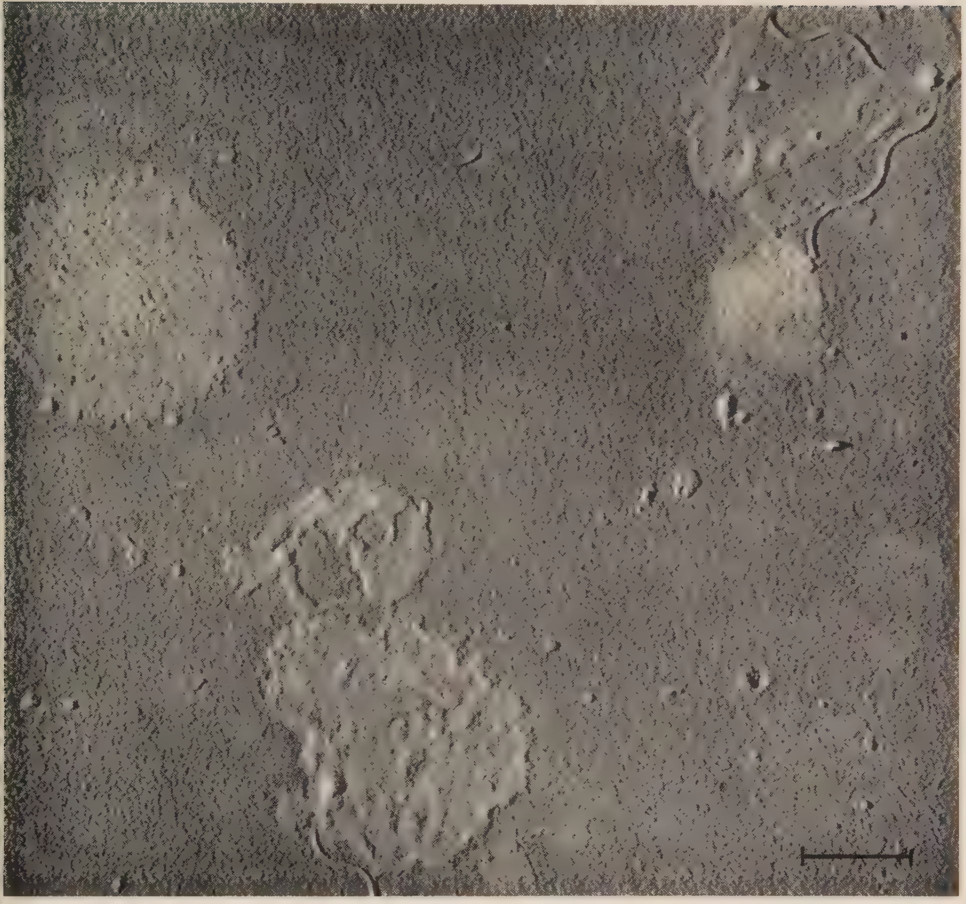


Fig. 4.—Electron micrograph of an air dried preparation of bacterial membranes fixed in osmic acid vapours for five minutes, and shadowed with metallic platinum.

18 per cent of the total dry weight of the cell. This value decreases to 12 per cent upon washing with distilled water. The protoplasts contain 60 per cent of the dry weight of the cell, and if one assumes 10–15 per cent protoplast rupture during formation, this leaves a value of 30 per cent for the cell wall dry weight. The dry weight of the membrane prepared by osmotic lysis of protoplasts is about 15 per cent, thus confirming the weight obtained by direct lysis of whole cells.

The photomicrographs of whole cells, protoplasts, and membranes are of hanging drop preparations viewed through phase contrast optics with dark

M objective. The cells in Fig. 1 are log phase cells in characteristic cell division. Fig. 2 depicts protoplasts suspended in 0.33 *M* sodium chloride. The appearance of the protoplasts depends, in part, on the tonicity of the medium, and the time elapsed subsequent to treatment. Thus it is quite possible to obtain a structure which resembles whole cells in every respect when viewed under the optical microscope, but which is susceptible to rapid lysis on resuspension in distilled water in the same way as are spherical protoplasts. Fig. 3 represents the membranes obtained on suspension of the protoplasts in distilled water. While it is not clearly evident in the print, this fraction is made up of the large spherical membranes and some small spherical granules or particles.

Fig. 4 is an electron micrograph of an air dried suspension of the membrane fraction mounted on Formvar covered 200 mesh grids. The membranes appear as flattened bodies with little rigidity of structure. *A. vinelandii* is a peritrichous organism [3], and this fraction contains numerous flagella. Examination of many membrane fraction preparations indicates the presence of some particulate material which in general is not directly associated with the membranes.

SUMMARY

The data reported here show that iron occurs predominantly in the membrane of *Azotobacter vinelandii,* while molybdenum and its antagonist tungsten are intracellular constituents. Although the evidence for the localization of calcium by these methods is not unequivocal, it appears that this metal may be found largely in cell wall material.

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Experimental Cell Research **15**, *85–94* (*1958*)

CONTROL OF DIFFERENTIATION IN THE FERN GAMETOPHYTE BY AMINO ACID ANALOGS AND 8-AZAGUANINE

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Received October 15, 1957

In the majority of *Filicales* ferns, especially in those belonging to *Polypodiaceae,* unicellular spores differentiate first into one rhizoidal and one protonema cell at germination. Then the protonema cell enters in a phase of successive cell divisions which occur in one direction, resulting in so called filamentous protonema. This growth pattern will be referred to as "the primary one-dimensional growth". After a definite time lapse, the direction of cell division of the apical cell of the protonema is converted from longitudinal to transverse. The subsequent division of these cells takes place in all directions but in the same plane, forming a plate-like tissue of monocellular layer. This growth pattern will be called "two-dimensional". The plate-like organism in the two-dimensional growth, i.e. young prothallium, develops first to a heart-shaped prothallium, and finally to a mature prothallium provided with antheridia and archegonia.

In *Dryopteris erythrosora* which has been studied in the present paper, spores begin to germinate five days after dusting in the culture medium under standard conditions. The primary one-dimensional growth of the protonema proceeds up to a seven-cell stage, which is followed by the two-dimensional growth beginning approximately 25 days after germination. About 100 days are required until the mature prothallium is completed.

The experiments to be described in this paper concern the artificial control of differentiation of this organism by some chemicals which are known to interfere with the synthesis of protein and nucleic acid.

MATERIAL AND METHOD

Spores of *Dryopteris erythrosora* were dusted in the culture medium in Petri dishes which were then placed under diffused day light and kept at 27°C.

As the standard culture medium, a modified Knop's solution was used: 2.32 g KH_2PO_4; 2.994 g Na_2HPO_4; 0.2 g $Ca(NO_3)_2$; 0.024 g KCl; 0.05 g $MgSO_4$ and $FeCl_3$ trace, made up to 1000 ml with glass-distilled water. The pH of the solution was kept at 6.3. The culture medium was refreshed every week and the morphological changes of protonema or prothallium were checked every fifth day.

Changes in the absolute amount of dry weight or in the protein nitrogen content of a single spore during the course of development was estimated in the following way: Eleven cultivations of spores in separate Petri-dishes were made under our standard conditions, each of cultures containing 1 mg of spores. The content of each dish was quantitatively collected successively 0, 10, 20, 25, 40, 50, 60, 70, 80, 90, and 100 days after the germination, and analysed for dry weight or protein nitrogen. It was found that 1 mg portion contains about 5500 spores, and dry weight or protein nitrogen content derived from single spore was calculated accordingly.

For the estimation of protein nitrogen, materials were collected from one to several Petri dishes at a desired stage. All collected material was carefully washed several times with distilled water, blotted, weighed, and kept in 80 per cent ethanol for six hours. After centrifugation it was homogenized with a glass homogenizer and suspended in cold 10 per cent trichloroacetic acid (TCA) solution and left undisturbed for 15 hours at 2°C. The precipitate obtained was suspended in 5 per cent TCA and heated at 90°C for 20 min. to remove nucleic acids. The precipitate washed with 5 per cent TCA was employed as the protein fraction. For the nitrogen estimation, a modified Levy and Palmer's method [7] was used.

RESULTS AND DISCUSSION

Changes in protein contents.—The concept that differentiation of the cell or tissue involves the synthesis of new kind of protein has now been generally accepted. It may therefore be of interest to know if the onset of the two-dimensional growth is characterized by a change in the protein metabolism.

In the first place, changes in the protein content during the formation of the young gametophyte have been followed. Representative data are shown in Fig. 1. From this it is clear that up to the end of the primary one-dimensional growth, only a slight increase in the absolute amount of protein is detectable. As soon as the two-dimensional growth begins, a sudden increase in the protein content becomes apparent which continues throughout the two-dimensional growth. As an accurate cell count in later stages of the two-dimensional growth is technically not possible at present, an estimation of the protein content per cell was not undertaken. However, if the protein content per dry weight is taken as an approximate index of the protein concentration in the cell, the one-dimensional growth is characterized by slightly decreasing protein concentration, whereas the two-dimensional growth is accompanied by a rapid increase in protein concentration.

As seen in the later section of this paper, such relationship between the protein concentration and the type of growth holds true when the mode differentiation is artificially altered.

Effects of amino acid analogs.—In the foregoing section, it was mentioned that the two-dimensional growth is characterized by a distinctly higher rate of protein synthesis. It may therefore be expected to have a preferential inhibition of the two-dimensional growth by affecting the synthesis of protein characteristic for it. For this purpose, the effect of amino acid analogs, ethionine and 5-methyltryptophane, was tested. In a preliminary experiment, each analog was introduced into the culture in a wide range of concentration ($M/50$ to $M/5000$). The developmental stages, at which analogs were added, involved spores, protonema of five-cell stage, and young prothallium 10 days after the two-dimensional differentiation. The morphological changes were followed under the microscope for about 50 days after the addition of analogs. Almost no effect on the morphological features was observed at a concentration below $M/1000$ for both analogs. Concentrations above $M/100$ cause

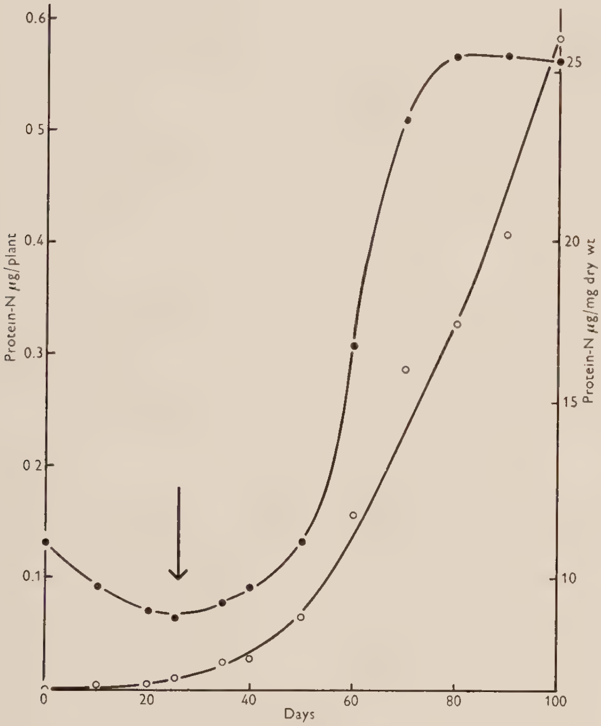

Fig. 1.—Changes in protein content per dry weight (●——●) and per single plant (○——○) during the course of germination and prothallium formation in *Driopteris erythrosora*. Arrow indicates the beginning of the two-dimensional growth.

death of organism shortly after the addition. At the concentration of about *M*/500 preferential inhibition of the two-dimensional growth was clearly demonstrated without any effect on the one-dimensional growth, and the two-dimensional growth was always converted into the one-dimensional growth.

When the analogs were added to the culture of ungerminated spores, germination and the subsequent primary one-dimensional growth were not affected. However at the seven-cell stage, when in the control the beginning of the two-dimensional growth is perceptible, no sign of the latter was noted in the experimental culture containing analogs. The apical cell kept proliferating in the one direction, forming a long filamentous protonema even when examined 50 days after the germination.

A comparable result was obtained when the analogs were added 10 days after the two-dimensional differentiation: the apex of the young plate-like prothallium at this stage is composed of three to six cells (only these cells proliferate under our experimental conditions), and by addition of analogs these apical cells changed the growth pattern from the two-dimensional to one-dimensional type.

As already mentioned, during the primary one-dimensional growth, no increase, but a slight decrease in the protein content per dry weight was noted. It would therefore be reasonable to assume that the "artificial" one-dimensional growth induced by amino acid analogs is characterized by the protein metabolism similar to that of the primary one-dimensional growth. To test this idea, the following experiments were performed. Ethionine or 5-methyltryptophane (*M*/500) was added in the culture containing the prothallium 10 days after the two-dimensional growth; after 15 days all cultures were found to contain long filamentous protonemata growing in the one-dimensional direction. Comparison of its protein concentration with those of the initial prothallium and with those of the young plate-like prothallium cultured for the same period without adding analogs (i.e. the control) made it clear that in the control a sharp increase in the protein content occurs, whereas in the culture containing analogs there is a slight decrease in the protein content (Table I). A tentative conclusion from this observation is that the decrease of the protein content is one of the characteristics of the one-dimensional growth whether it is normal or artificial.

The next experiment was arranged to show a reversibility of the inhibition preferentially induced on the two-dimensional growth by the analogs. It was found that the simultaneous addition of the same concentration of methionine or tryptophane respectively to ethionine or 5-methyltryptophane com-

TABLE I. *Effects of ethionine and 5-methyltryptophane on the differentiation and the protein content of young prothallium of* Dryopteris erythrosora.

For explanation, see text.

	Starting material	Control	Experimental	
			Ethionine	5-Methyl-tryptophane
Protein-*N*: μg/mg dry wt.	8.9	11.2	8.2	7.8
Type of growth	—	Two-dimensional	One-dimensional	

pletely compensates the action of these analogs on the morphological differentiation. In these experiments simultaneous administration was done on the cultures of spore, protonema of five-cell stage, and young prothallium 10 days after the two dimensional differentiation.

If methionine or tryptophane was added to the culture in which the "artificial" one-dimensional growth induced by amino acid analogs was going on, a complete reversal of the one-dimensionl to the two-dimensional growth was observed. In such experiments the amino acid analogs were first added to the culture containing organisms at the developmental stages mentioned above, and after 14 days methionine or tryptophane was introduced respectively.

Effect of 8-azaguanine.—As it is generally accepted that nucleic acids are involved in the protein synthesis, the question may be raised whether the intense protein synthesis which accompanies the two-dimensional differentiation is connected with nucleic acids. Attempts to estimate the nucleic acid content in our material have not been so far successful. We tried a number of procedures, which all failed to estimate reasonable values of both nucleic acids because of the presence of some substances which vigorously interfere with the ultraviolet adsorption at 260 mμ, color reactions of sugars, and phosphorus determination of nucleic acids. It was therefore designed to use the substance which selectively suppresses the nucleic acid metabolism.

8-Azaguanine is known to be the most effective inhibitor of synthesis of ribonucleic acid (RNA). It is already shown that this substance actually incorporates into RNA in place of guanine residue, forming "abnormal RNA" which interferes with the subsequent growth of organisms in the case of *Tetrahymena geleii* [2], tobacco mosaic virus [3], growing culture of bacteria

[2, 3, 5], and some animal tissues [2, 4]. Incorporation of 8-azaguanine into deoxyribonucleic acid is commonly very little or insignificant [1, 2, 3]. We have tested the effect of 8-azaguanine on the growth pattern of the fern gametophyte. To find the optimum concentration of this substance which might selectively affect the two-dimensional growth, concentrations from *M*/500 to *M*/25,000 were applied to the culture medium. Stages used in this test involved spores, the prothallium at five-cell stage, the young plate-like prothallium a few days after the two-dimensional differentiation, and 50 days old prothallium. At a concentration below *M*/17,500 no morphological effect was noted by 8-azaguanine. The substance above *M*/1000 caused gradual or immediate death depending on the concentration applied. However in the concentration range between *M*/12,000 and *M*/14,000 a preferential inhibition of the two-dimensional growth was clearly observed. As the results obtained were comparable with those found in the case of amino acid analogs, they are not repeatedly described here.

The next experiment is to show that the specific inhibitory effect of 8-azaguanine at the concentration of *M*/12,500 could be obtained at any developmental stage. 8-Azaguanine was added to the culture of spore, protonema of five-cell stage, prothallium a few days after the two-dimensional differentiation and prothallium 50 days old; its effect was examined 20 days and 30 days after the addition. Here again, at each stage tested, upon addition of 8-azaguanine the two-dimensional growth was completely suppressed and converted into the one-dimensional, while the one-dimensional growth was not affected.

Alleviation of the growth inhibition by 8-azaguanine was achieved by the addition of the same concentration of guanine. When the guanine was added simultaneously with 8-azaguanine and tested on spore, protonema of five-cell stage, young prothallium a few days after the two-dimensional differentiation, prothallium 50 days old, no inhibitory effect on the two-dimensional growth was observed.

If guanine was added to the culture in which the "artificial" one-dimensional growth was taking place in the presence of 8-azaguanine, a complete reversal of 8-azaguanine effect became apparent immediately after the addition of guanine. In this series of experiments 8-azaguanine was first added to the culture containing five different stages of development and after 14 days the same amount of guanine is introduced. Morphological appearance of organisms in each culture ten days after guanine introduction is schematically illustrated in Fig. 2. Although no data on the behavior of nucleic acid in this experiment are available at present, the results mentioned above strongly suggest that 8-azaguanine inhibits the two-dimensional growth by interfering with the RNA metabolism. The fact that both amino acid analogs and nucleic acid antagonist cause the same inhibitory effect on two-dimensional growth would indicate that the specific RNA synthesis sensitive to 8-azaguanine is necessary for the formation of protein characteristic for the two-dimensional growth, which is sensitive to the amino acid analogs. This being the case, it might be expected that the formation of protein detect-

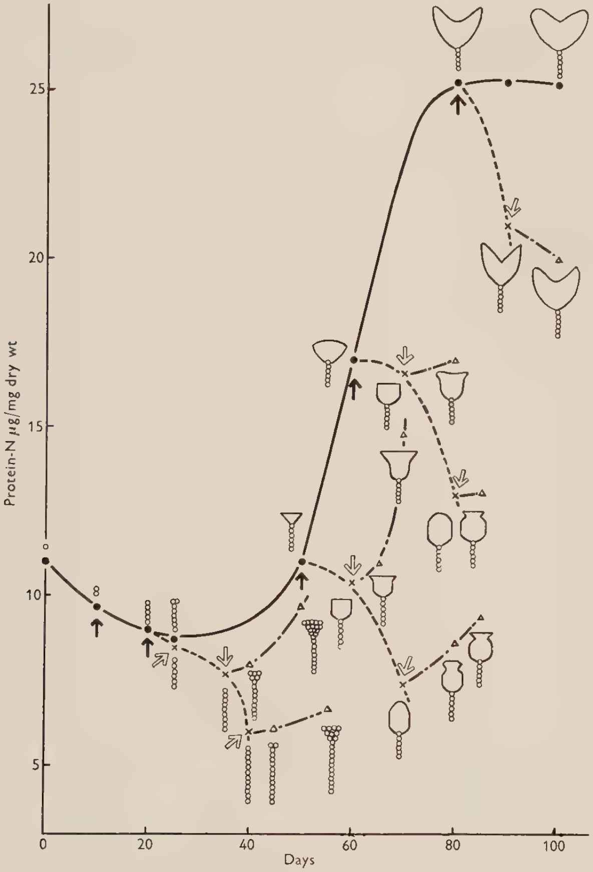

Fig. 2.—Effect of 8-azaguanine on the differentiation and protein content of young prothallium of *Driopteris erythrosora* with the reversal of 8-azaguanine effect by the addition of guanine. O——O: protein-*N* per mg dry weight in control culture; ×——×: protein-*N* per mg dry weight in the cultures to which *M*/12500 8-azaguanine was added at the time indicated by black arrow; ▲——▲: protein-*N* per mg dry weight in the cultures to which first 8-azaguanine was added, followed by the introduction of the same concentration of guanine at the time indicated by white arrow. Appearance of prothallium was schematically presented at each point.

able during the two-dimensional growth is completely inhibited by 8-azaguanine. To test this, 8-azaguanine was added at the concentration of *M*/12,500 in the culture containing spore, protonema of five-cell stage, prothallium 50 days old, 60 days old, and 80 days old. All cultures were allowed to develop for 5 to 15 days only in the one-dimensional growth pattern. In Fig. 2 the data of protein estimation of such cultures are compared with those of the corresponding controls in which no 8-azaguanine had been introduced. The results clearly demonstrate that 8-azaguanine causes a decrease in protein concentration and simultaneously a complete inhibition of prothallium formation characteristic for the two-dimensional growth. The situation is exactly comparable to the case of amino acid analogs. Upon introducing guanine after five and fifteen days after 8-azaguanine addition, a marked increase of protein concentration was demonstrated with a simultaneous commencement of the two-dimensional growth. The results of this experiment are also included in Fig. 2.

Effects of general metabolic inhibitors.—The fact that the two-dimensional growth (and the accompanied protein synthesis) is completely inhibited by interfering either with protein synthesis or with the RNA metabolism is indicative of these processes being characteristic of two-dimensional growth. There are at least three ways of interpreting those results:

(1) Two-dimensional growth requires a certain level of protein synthesis which is qualitatively not different from that of one-dimensional growth. 8-Azaguanine and amino acid analogs cause non-specific lowering of the synthetic rate of protein below to the level, thus resulting in the conversion of the mode of growth.

(2) Two-dimensional growth is causally connected with the formation of a distinct protein(s) which is specifically inhibited by 8-azaguanine and amino acid analogs.

(3) Two types of growth in the problem are preceded by a combination of two (or more) metabolic processes which are qualitatively common in both. One-dimensional growth is brought about by a lower level of protein synthesis and a predominancy of another metabolic activity. On the other hand two-dimensional growth is supported by a higher level of protein synthesis plus a lower activity of another process. Thus the lowering of protein synthesis by 8-azaguanine and amino acid analogs caused the competitive increase in another activity. As a result, the mode of growth is converted from two-dimensional to one-dimensional.

Now, if the first explanation is correct, then it would follow that the interference of general metabolism such as respiration, phosphorylation, and glycolysis (which result from the lowering of the protein synthesis) may equally result in an inhibition of the two-dimensional growth without affecting the one-dimensional growth. On the other hand, if the second or third process is involved, the introduction of a general inhibitor would cause only non-specific interference of growth in its effective range of concentration without preferential inhibition of the two dimensional growth.

In the first series of experiments, spores were allowed to germinate in the presence of inhibitors in a wide range of concentrations (10^{-2} *M* to 10^{-14} *M*). The inhibitors used were KCN, NaN_3, 2,4-dinitrophenol, NaF, and sodium malonate. Those inhibitors either caused a simple inhibition of growth, or were without effect. No inhibitor at any concentration tested could cause a selective inhibition of the two-dimensional growth, or a conversion from the two-dimensional to the one-dimensional growth.

The second series of experiments were carried out so that the inhibitors mentioned above were applied to the culture containing the young plate-like prothallium in the two-dimensional growth (seven days after inception of the two dimensional growth). In no case was the preferential inhibition of the two-dimensional growth obtained: the inhibitors caused either death or a general retardation of growth depending on the concentration of inhibitors applied. In lower concentrations the growth and differentiation were not affected at all.

Whether the protein synthesized at the time of the two-dimensional growth is of a distinclty specific nature or not must be left open for the time being. However, the fact that a general inhibition of the cellular metabolism does not cause selective inhibition appears rather to favor an involvement of specific protein formation which is selectively affected by amino acid analogs or 8-azaguanine.

As for the mode of action of those inhibitors, we do not have direct evidence. One possibility is that the formation of RNA is indispensable as a component of protein synthesizing machinery, and that 8-azaguanine incorporates into RNA a moiety of this machinery in place of the guanine residue, rendering it inert. However, we have no evidence for the incorporation of the added 8-azaguanine into RNA. An experiment is now in progress in which the uptake of C^{14}-8-azaguanine into RNA under our experimental conditions is being tested.

Amino acid analogs might be directly incorporated in the postulated specific protein as its synthesis occurs, so that the two-dimensional growth is inhibited. However it is equally possible to assume that analogs incorporate into the protein synthesizing machinery, and make it abnormal.

SUMMARY

Estimation of the protein contents during the course of development clearly indicates that whenever the two-dimensional growth takes place, a sharp increase in protein concentration in the cell can be observed, whereas in the course of the primary one-dimensional growth, protein concentration is gradually lowered. It has been shown that the two-dimensional growth can be specifically inhibited by some amino acid analogs such as ethionine and 5-methyltryptophane and it is converted to the "artificial" one-dimensional growth. The same selective interference of the two-dimensional growth is also brought about by 8-azaguanine, the effective inhibitor of the nucleic acid synthesis. Further it has been demonstrated that such inhibition can be alleviated by adding corresponding amino acids or guanine respectively.

Regardless of the nature of the effective inhibitors used, the selective inhibition is always accompanied by an immediate cessation of increase in the protein concentration, followed by its gradual decrease. The results strongly suggest that the two-dimensional growth is causally connected with the rapid synthesis of protein which is supported by the formation of RNA.

The senior author (Y. H.) is indebted to Prof. Tamaki Shimamura for his general guidance during the course of this study. His thanks are also due to Dr. Ichitaro Harada and Dr. Michio Ito for their continuous interest and help.

We express our sincere gratitude to Prof. Tuneo Yamada for his advice and critical reading of manuscript.

This work is supported by grants from the Rockefeller Foundation, and from the Ministry of Education for Fundamental Scientific Research awarded to "Cooperative Research on the Nucleic Acids".

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Experimental Cell Research **15**, *95–102* (*1958*)

THE CYTOCHROME SYSTEM IN MITOCHONDRIA OF UNFERTILIZED SEA URCHIN EGGS

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Received October 15, 1957

THE existence of a cytochrome system in sea urchin eggs was first indicated by Runnström [16] on the basis of carbon monoxide inhibition of the respiration. Direct evidence of the existence of the cytochrome system in sea urchin eggs was given by the spectroscopic observation of eggs frozen at the temperature of liquid air. The spectrum, however, appeared to be atypical as bands *b* and *c* were replaced by a single band, b_1 [15].

The presence of a cytochrome oxidase and of cytochrome *c* was further demonstrated by spectroscopic and enzymatic tests in intact cells, cell-free extracts and acetone powders [1, 2, 3, 10, 17].

However, the question whether the terminal electron transfer to molecular oxygen in sea urchin eggs is the same as in vertebrates, remained open on account of several negative results, i.e. carbon monoxide failed to inhibit the respiration of unfertilized eggs [17]; the cytochrome *c* was found only in trace amounts [1, 2, 3, 10]; the succinic oxidase activity was absent from both unfertilized and fertilized eggs [6].

These negative results suggested the idea that the cytochrome system, even if present, was not operating in the terminal respiration of the unfertilized sea urchin eggs [8].

Very recently a method for obtaining mitochondrial preparations from sea urchin eggs has been described [13]. By using such suspensions we have been able to demonstrate the presence in unfertilized sea urchin eggs of a complete and active cytochrome system which appears to be very similar to that of mammals and yeast.

MATERIAL AND METHODS

Mature unfertilized eggs of *Paracentrotus lividus* and *Sphaerechinus granularis* were used. The eggs were separated from the gonads by gentle shaking in sea water and filtered through bolting silk. They were then centrifuged at low speed for 2 minutes and the jelly coat removed by a rapid treatment with acidified sea water (pH about 5). Further manipulations were performed at 4°C.

After homogenization of the packed eggs in a Potter-Elvehjem glass homogenizer with a teflon pestle, the mitochondria were prepared, in the case of *Paracentrotus*, by the method described by Monroy [13]. In the case of *Sphaerechinus* eggs the preparation was essentially the same except that the eggs were homogenized in 0.5 *M* sucrose containing 5×10^{-4} *M* versene at pH 7.3 and the last washing was made in 0.8 *M* sucrose.

In some cases the mitochondrial preparation was further purified by a differential centrifugation in a density gradient of sucrose [12]. 1.5 ml of the original suspension in 0.5 *M* sucrose were layered over the contents of cellophane tubes containing from bottom to top 1.5 ml each of 1.5 and 1.0 *M* sucrose. After 1 hour at 30,000 rpm in the SW-39 rotor of the Spinco preparative ultracentrifuge, the tubes were cut with the special cutter described by Randolph and Ryan [14] and the layers collected separately for further determinations.

The activity of the enzymes was determined in a conventional Warburg apparatus at 24°C. Cytochrome oxidase and succinic oxidase activity were determined according to Slater [19, 20]. The inhibition of the cytochrome oxidase by carbon monoxide and its reversibility by light was studied according to Warburg [22]. The vessels containing the complete system were saturated for 10 minutes with a mixture of CO and O_2 (95:5 per cent), while in the control vessels a mixture of N_2 and O_2 (95:5 per cent) was used. For the study of the photoreversibility of CO inhibition, the light source was a Philips neon lamp fitted for underwater use and placed in the water bath directly below the vessels. Carbon monoxide was prepared from formic acid and hot sulfuric acid. Cytochrome *c* was prepared from beef heart muscle according to Keilin and Hartree [8]. Nitrogen was determined colorimetrically by direct nesslerization after oxidation.

The difference spectra of the cytochrome system and of the CO compound of cytochrome oxidase were taken with a DU Beckman spectrophotometer equipped with a photomultiplier attachment according to the method previously described [5].

RESULTS

Cytochrome oxidase activity.—Cytochrome *c* being water soluble is readily lost during preparation of the particulates. Since it is the limiting factor in the determination of cytochrome oxidase activity, addition of reduced cytochrome *c* was found to be necessary for the manometric assay of the oxidase activity. The increment of oxygen uptake so obtained was taken as a measure of the enzyme activity.

Table I shows the effect of cytochrome *c* concentration on the cytochrome oxidase activity of the mitochondrial preparations. Blanks without cytochrome *c* gave a negligible oxygen consumption. Saturation was reached with 3.26×10^{-5} *M* cytochrome *c*. Higher concentrations gave a slight but consistent decrease of the oxygen uptake.

In Table II the activity at different enzyme concentrations is reported. The incomplete proportionality of the oxygen uptake which has been observed

TABLE I. *The effect of cytochrome c concentration on the cytochrome oxidase activity.*

The vessels contained: mitochondrial suspension in phosphate buffer 0.033 *M*, pH 7.3, 0.01 *M* sodium ascorbate (final concentrations) and different amounts of cytochrome *c*. Gas phase air, $t = 24°C$. KOH in the central well.

μl O_2/hr/mg protein.

Cytochrome *c* × 10^{-5} *M*	*Sphaerechinus*	*Paracentrotus*
0.41	97.0	—
0.82	151.6	—
1.64	284.6	207.4
2.46	339.5	267.7
3.28	340.5	279.7
4.10	273.5	273.8

TABLE II. *The effect of enzyme concentration on the cytochrome oxidase activity.*

The system contained: 0.033 *M* phosphate buffer pH 7.3, 0.01 *M* sodium ascorbate, 2.46 × 10^{-5} *M* cytochrome *c* (final concentration) and different amounts of mitochondrial suspension. Gas phase: air, $t = 24°C$.

	Enzyme mg protein	μl O_2/hr
Sphaerechinus	0.258	67.6
	0.516	106.4
	0.774	149.0
Paracentrotus	0.325	88.4
	0.650	158.8
	0.975	252.4
	1.300	307.2

might be due to a non-complete saturation of the enzyme with cytochrome *c*. In order to avoid inhibition at higher concentrations, the quantity of cytochrome *c* added was always just enough to saturate the minimum amount of the enzyme used.

The optimum pH was found to be around 7.1. The enzyme activity did not change with the partial oxygen pressure in the gas phase.

Fig. 1 shows the inhibition by CO in dark and light of the cytochrome oxidase activity of *Sphaerechinus* preparations. An inhibition of 80 per cent was observed in the dark; it was completely reversed by light. With *Paracentrotus* preparations the inhibition was found to be 90 per cent and complete reversibility by light was also observed.

Succinic oxidase activity.—The succinic oxidase activity, as measured manometrically, was found to be very low in mitochondria of unfertilized sea urchin eggs. One of the most active preparations had a Q_{O_2} (μl O_2/mg protein/hr) of 14; addition of beef heart cytochrome *c* 0.82×10^{-5} *M* gave a value of 32. In all cases the experiments were run for periods no longer than 30 minutes. Using 1 mg/ml of protein and 0.82×10^{-5} *M* cytochrome *c*, the enzyme activity was found to be proportional to the substrate concentration up to 3.3×10^{-2} *M* succinate; the optimum pH was around 7.3.

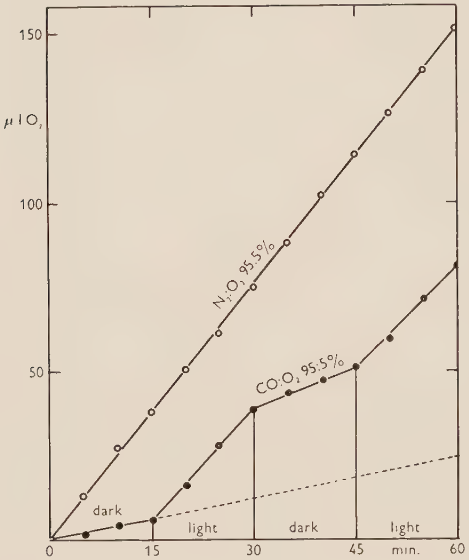

Fig. 1.—Mitochondria from eggs of *Sphaerechinus granularis.* The effect of alternate darkness and illumination on carbon monoxide inhibition of the cytochrome oxidase activity. The vessels contained: 0.033 *M* phosphate buffer pH 7.3, 0.01 *M* sodium ascorbate, 2.46×10^{-5} *M* cytochrome *c* and 0.15 mg/ml protein (final concentrations). $t = 24$°C. *Abscissa,* time in minutes; *ordinate,* μl O_2.

Difference spectra.—All the difference spectra were taken on mitochondria suspended in 0.1 *M* phosphate buffer pH 7.3. Reduction was obtained by addition of succinate or ascorbate or dithionite.

Fig. 2 shows the spectra obtained for *Sphaerechinus* mitochondria. Since reduction by succinate is quite slow, a 30 minutes period was necessary for the cytochromes to be fully reduced. When succinate is used (solid line) the spectrum shows, in the Soret region, two distinctly separate peaks at 430 mμ and 444 mμ corresponding to the γ band of cytochrome *b* and cytochrome a_3 respectively. The γ band of cytochrome *c* should appear at 415 mμ; this band is not visible owing to the low relative concentration of this compound in the washed mitochondria [21]. In the visible region of the spectrum three bands are present. The band at 520 mμ corresponds to the β band of cytochrome $b + c$; the bands at 562 mμ and at 605 mμ are the α bands of cytochrome $b + c$ and of cytochrome a respectively. The trough at 470 mμ corresponds to the reduced flavins.

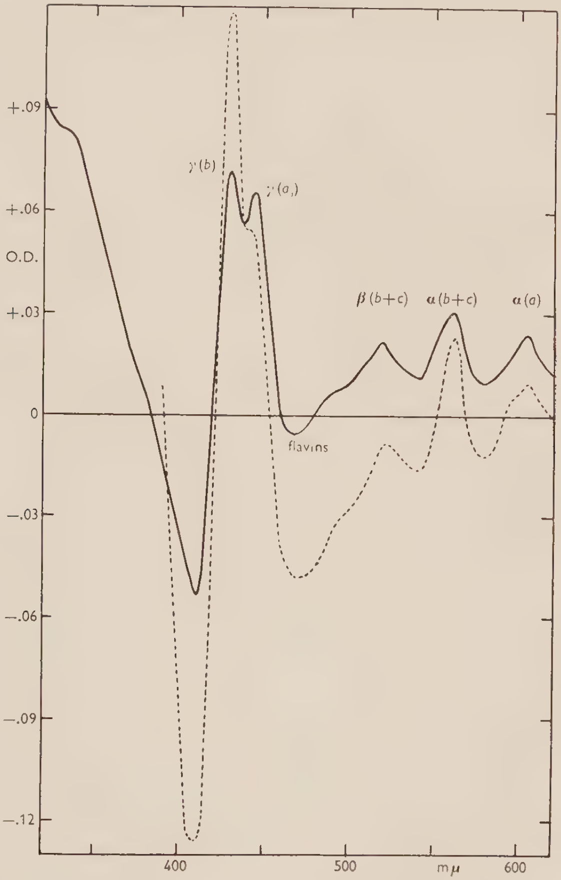

Fig. 2.—Difference spectra (reduced minus oxidized) of a mitochondrial suspension from eggs of *Sphaerechinus*. Solid line: reduction with succinate; dotted line: reduction with dithionite. Readings were taken at intervals of 10 mμ except for the regions around the peaks where readings were taken at 2 mμ intervals. *Abscissa*, wavelength; *ordinate*, optical density.

By addition of dithionite (dotted line) an increase of the γ band of cytochrome b is observed; this is an indication that not all of the cytochrome b present has been reduced by succinate. In this spectrum the γ band of cytochrome a_3 appears as a shoulder at 444 mμ.

As it can be seen from Fig. 2, after reduction with dithionite, the base line is lowered in the visible region of the spectrum. Mitochondrial preparations from sea urchin eggs contain some non-respiratory pigments which are also reduced not only by dithionite but also by more specific agents like succinate and ascorbate. This fact is more apparent in *Paracentrotus*, whose eggs have a deep orange-red colour which cannot be removed during the normal preparation of the mitochondria. While trying to remove this pigment, the original mitochondrial suspension was further centrifuged in a density gradient of sucrose. Three different fractions were obtained, one of which was still deep orange-red, one pale-yellow and the heaviest pink. They were collected separately and observed spectrophotometrically.

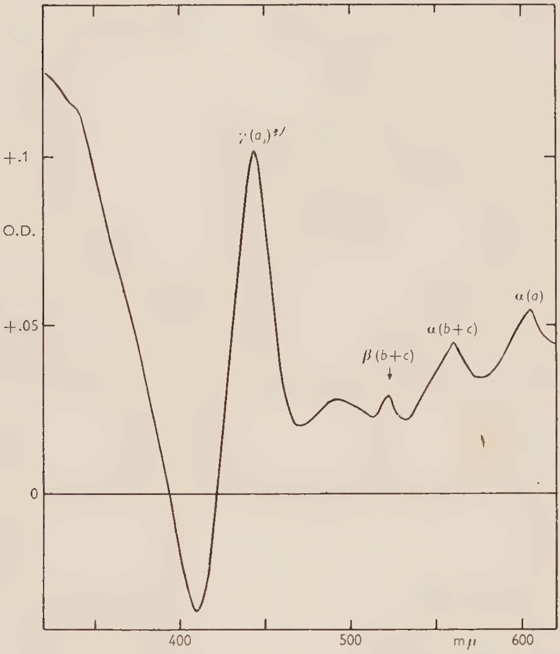

Fig. 3.—Difference spectrum (reduced minus oxidized) of a mitochondrial fraction obtained by differential centrifugation in a density gradient of sucrose, from eggs of *Paracentrotus lividus*. Reduction with succinate. Readings were taken at the same intervals as in Fig. 2. *Abscissa*, wavelength; *ordinate*, optical density.

Unexpectedly, only the orange-red fraction, which seemed to be formed mostly of fats, gave a good differential spectrum which is reported in Fig. 3. In this spectrum most of the bands already described for the *Sphaerechinus* preparation are present. The γ band of cytochrome *b* is not clearly visible even after reduction with succinate. Yet, the presence of cytochrome *b* is indicated not only by the peak at 560 mμ, but also by the reduction of cytochrome a_3 obtained by the addition of succinate.

For the spectral study of the carbon monoxide-cytochrome oxidase compound the original mitochondrial suspension was treated with ascorbate and its spectrum was read against the oxidized form. Reduction with ascorbate gave the same result as with succinate except that the bands of cytochrome *b* were not clearly visible. Later also the oxidized form was treated with ascorbate and then saturated with CO. The ascorbate-CO compound was read against the first ascorbic acid treated suspension. A shift at 430 mμ of the former peak at 444 mμ and a trough at 445 mμ were observed, both characteristic of the spectrum of the CO-cytochrome oxidase compound.

CONCLUDING REMARKS

Mitochondria prepared from unfertilized sea urchin eggs contain a complete cytochrome system formed by cytochromes a, a_3, b and c. This is demonstrated by the difference spectra of the respiratory pigments (reduced minus oxidized) and by the succinic and cytochrome oxidase activity of the mitochondrial suspensions. The Soret band of cytochrome is not visible in the difference spectra; this water soluble pigment has been mostly removed during the preparation of the mitochondria. However, indirect evidence of the presence of cytochrome c in sea urchin eggs mitochondria is given by the appearance of the reduced bands of cytochrome a and a_3 when specific reducing agents, like succinate, are used. This indicates that its concentration in mitochondria, even if very small, is still sufficient to operate the electron transfer from cytochrome b to cytochrome oxidase.

The cytochrome system present in unfertilized sea urchin eggs is very similar to that described in mammals, the bands of the cytochromes in the difference spectra having peaks at the very same positions as those observed for mammalian cytochromes [21]. Moreover, as shown by manometric experiments, sea urchin cytochrome c can be replaced by cytochrome c from beef heart muscle. Cytochrome oxidase is also very similar to the mammalian oxidase. The peak at 430 mμ and the trough at 445 mμ observed in the difference spectra of the carbon monoxide-cytochrome a_3 compound of mitochondria from sea urchin eggs correspond exactly to those known for yeast and mammals [4, 9, 11, 23, 24].

It seems interesting to draw attention to the finding that the fractionation of the particulate sediment in a density gradient has shown that the cytochrome system is to be found only in the pigment-containing fraction. According to current views, the main character of the mitochondria is their being the site of the cytochrome system; hence, in the present case, only the pigment-containing granules should be spoken of as mitochondria. On the other hand, adenosinetriphosphatase, which is another mitochondrial enzyme, has been found both in the pigment-containing and in the pigment-free granules (Ricotta, personal communication), although the activities of the two categories of granules differ in their pH optimum for Mg-activation. These observations indicate the heterogeneous character of the particulate population of the sea urchin egg. It may provisionally be suggested that during some part of their life cycle the mitochondria of the sea urchin egg store carotenoid pigments which most likely play some yet unknown role in their physiological activity. A similar suggestion has been recently presented by Gustafson [7].

SUMMARY

1. The cytochrome system of unfertilized sea urchin eggs has been studied using preparations of isolated mitochondria.

2. The difference spectra of isolated mitochondria show the presence of a complete and operating cytochrome system formed by the cytochromes a, a_3, b and c.

3. This cytochrome system is very similar to that of mammals and yeast as demonstrated by spectrophotometric and manometric experiments.

This work has been supported in part by a Grant from Consiglio Nazionale delle Ricerche to the Laboratory of Comparative Anatomy, University of Palermo. One of us (A. G. M.) is under the tenure of a Grant (RG-4548) of the U.S. Public Health Service.

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Experimental Cell Research **15**, *103–111* (*1958*)

INTRACELLULAR STRUCTURES IN *STRIGOMONAS ONCOPELTI*

II. FINE STRUCTURE OF THE KINETOPLAST–BLEPHAROPLAST COMPLEX

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Received October 17, 1957

The kinetoplast[1] is a characteristic cytological feature of trypanosomid lagellates; light microscopy has shown that it is probably a disc-shaped organelle (ca. 1 micron in diameter) which lies adjacent to the blepharoplast or basal granule. The blepharoplast is generally regarded as the starting point of the flagellum. There seems to be little doubt that these two structures are ntimately related; "strands" have been observed joining the two structures [25] and at the onset of cell division, when the kinetoplast migrates to a posiion close to the nucleus and cell membrane, the two structures remain adjacent. The kinetoplast is stained intensely by chromatinic dyes, whereas he blepharoplast is achromatic [25]. Numerous workers have demonstrated hat the kinetoplast contains Feulgen positive material at its periphery and achromatic material at its centre [2, 4, 14, 16, 25]; the peripheral material s also stained by methyl green and is digested by deoxyribonuclease [2]. Both the kinetoplast and the blepharoplast are self-duplicating structures, reproduction occurring by bipartition at the onset of cell division. The funcion of the kinetoplast remains unknown.

The work described in the present paper was undertaken in an attempt o learn something of the structural interrelationships existing between the lagellum, blepharoplast and kinetoplast in the trypanosomid flagellate *Strigomonas oncopelti*.

METHODS

Organism and growth media.—The strain of *Strigomonas oncopelti* described previously [21] was used. Stock cultures were maintained in 5 ml volumes of 3 per cent w/v) Evans Bacteriological Peptone containing 0.5 per cent (w/v) glucose and 0.5

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[1] Kinetoplast; this structure has, in the past, been called the kinetonucleus, centrosome, blepharoplast, parabasal body. The terminology has been fully discussed by Hoare [10] and by Kirby [15].

per cent sodium chloride, at an initial pH value of 7.4. The medium was sterilized by autoclaving at 15 lb/sq in. for 20 min. Cultures were incubated at 25°C and subcultured every other day. Larger batches of organisms were grown in 50 or 500 ml of the above medium contained in either 150 ml or 1 litre flasks; a 10 per cent inoculum from a 15 hr culture was used in all cases.

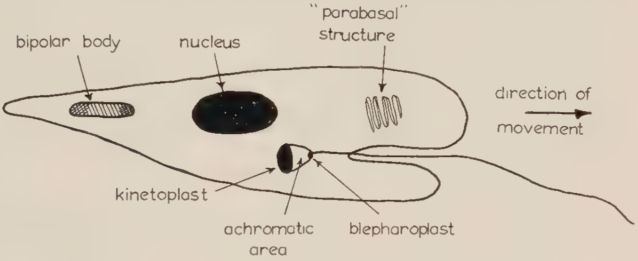
Cytochemical techniques.—Smears of organisms were prepared on microscope slides, dried in air and fixed by immersion in absolute methanol for 10 min. Fixed preparations were then stained with May-Grünwald-Giemsa dyes or with methyl green and pyronin as described by Jacobson and Webb [13]. As a control for the staining of deoxyribonucleoprotein by the above methods, preparations were stained by Feulgen's method after 3 min. hydrolysis in N HCl at 60°C. Some fixed smears were treated with deoxyribonuclease (Sigma Chemical Co., U.S.A.) before staining; incubations in solutions of enzyme (0.1 per cent w/v deoxyribonuclease in glass distilled water containing 0.075 M $MgSO_4$) were carried out at 37°C for varying periods of time. In each experiment a control preparation was incubated in glass distilled water alone.

Preparation of specimens for electron microscopy.—Intact organisms were fixed in 1 per cent buffered osmium tetroxide (pH 7.5) as described by Palade [22] and Porter and Kallman [24]. After fixation for periods ranging from 15–19 min. preparations were dehydrated by passing through a series of graded alcohols before being embedded in a mixture of 9 parts butyl- to 1 part methyl-acrylate; the embedding medium was polymerized in an oven at 45°C. Sections were cut and examined in the Siemens "Elmiskop" electron microscope operating at 80 Kv; instrumental magnifications of 5000 ×, 10,000 × and 20,000 × were used. It was found that a fixation time of 30 min. was the most satisfactory.

OBSERVATIONS

Light microscopy.—Light microscopy of stained preparations has established that the kinetoplast blepharoplast complex of *Strigomonas oncopelti* is similar in appearance and staining properties to that of other trypanosomid flagellates [2, 4, 14, 16, 25]. Its position within the cell is always anterior to the nucleus; the flagellum enters the cell through an invagination at the anterior end [17] as shown in Text-fig. 1. In preparations stained with May-Grünwald-Giemsa dyes the flagellum could be seen as a pale pink thread passing through the cytoplasm towards the densely stained kinetoplast; in all preparations examined the flagellum appeared to arise in an achromatic region ca. 1 micron from the kinetoplast and fine eosinophilic strands could often be seen connecting the point of origin of the flagellum to the kinetoplast. The kinetoplast was found to contain Feulgen positive and methyl-green positive material which could be removed by incubating preparations with deoxyribonuclease for 1 hr at 37°C before staining.

Electron microscopy of thin sections of Strigomonas oncopelti.—Fig. 1, an electron micrograph of a longitudinal section of *S. oncopelti*, shows the relative positions within the cell of a bipolar body (described previously [21]), the nucleus, kinetoplast and "parabasal structure". Figs. 2 and 2*a* are serial sections which show the kinetoplast area in greater detail and also a relatively long portion of flagellum in the cell which appears to come into contact with the kinetoplast. Transverse sections cut through flagella show the usual internal structure of nine peripheral and two axial fibres. The eleven fibres are surrounded by an outer sheath which is elongated on two sides to form ribs (Figs. 10 and 11); this structure is strikingly similar to that observed in animal spermatozoa tails [3]. Transverse sections cut through flagella within the cell have shown that the two axial fibres of the 9 + 2 arrangement may be missing (Fig. 9). Examination of many sections of *S. oncopelti* has suggested that the outer 9 fibres extend to the base of the kinetoplast and terminate in a dense granular area (Fig. 3). No evidence has been obtained for the presence of a second organelle, distinct from the kinetoplast, at the point of origin of the flagellum (i.e. the achromatic structure frequently observed in stained preparations and referred to as the blepharoplast). The dense granular area at the base of the kinetoplast is surrounded by a relatively electron-transparent area containing a complex network of fine filaments linking a large number of electron-dense granules (Figs. 3, 4, 5, 6 and 7). The diameter of the individual filaments in the sections examined varied from 50 Å to 150 Å. The number of granules was found to vary considerably depending upon the region of the kinetoplast sectioned. In certain transverse and longitudinal sections through kinetoplasts a peripheral area of electron-dense material was observed (Figs. 2, 2*a*, 4, 6 and 7), the appearance of which closely resembled that of nuclear material.

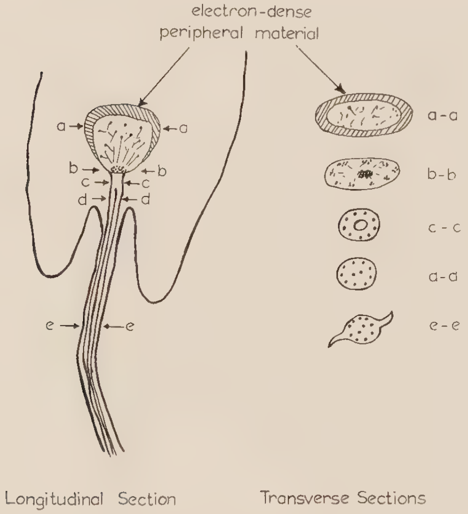


Text-fig. 1.—A diagram to show the relative positions of intracellular structures in *Strigomonas oncopelti* which have been observed by light and electron microscopy.

Fine membranous structures which resemble the "parabasal structures" observed in other protozoa [6, 8, 28] have been seen in a number of sections, but always in an area of the cell which is some distance away from the kinetoplast (Fig. 2).

DISCUSSION

The uniformity of internal structure (9 peripheral + 2 axial fibres) among cilia and flagella throughout the plant and animal kingdoms has been well established during recent years [3, 7, 9, 18]. The present work has shown that the flagellum of *Strigomonas oncopelti* conforms to this ubiquitous pattern and the observations described also give some indication of the complex relationship which exists between the flagellum and the kinetoplast of this organism. Text-fig. 2 summarises, in a diagrammatic form, data obtained from the study of more than 200 sections cut through the kinetoplast area of *S. oncopelti*. It is realised that many features in this diagram are highly speculative; they are intended only to form a basis for discussion and further work.



Text-fig. 2.—A speculative diagram based on electron microscope observations showing the structural features of the flagellum and kinetoplast of *Strigomonas oncopelti* and a possible interrelationship between them.

There would seem to be no doubt that the flagellum of *Strigomonas oncopelti* passes into the cell at an invagination at its anterior end [17]. At the point of entry into the cell all eleven fibres of the flagellum are present (Fig. 8),

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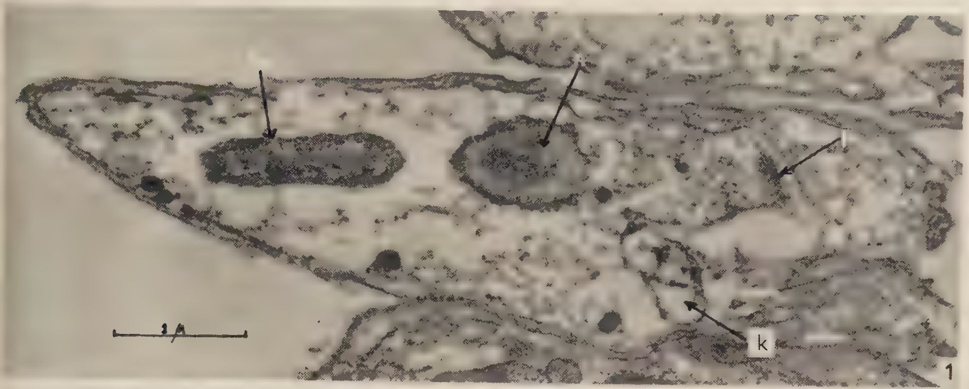
Fig. 1.—An electron micrograph of a longitudinal section of *Strigomonas oncopelti* showing kinetoplast (*k*), nucleus (*n*), parabasal body (*p*) and bipolar body (*bp*).

Fig. 2.—A longitudinal section showing the flagellum (*f*) within the cell and internal structure of the kinetoplast. Inset (Fig. 2*a*) is a serial section from the same organism.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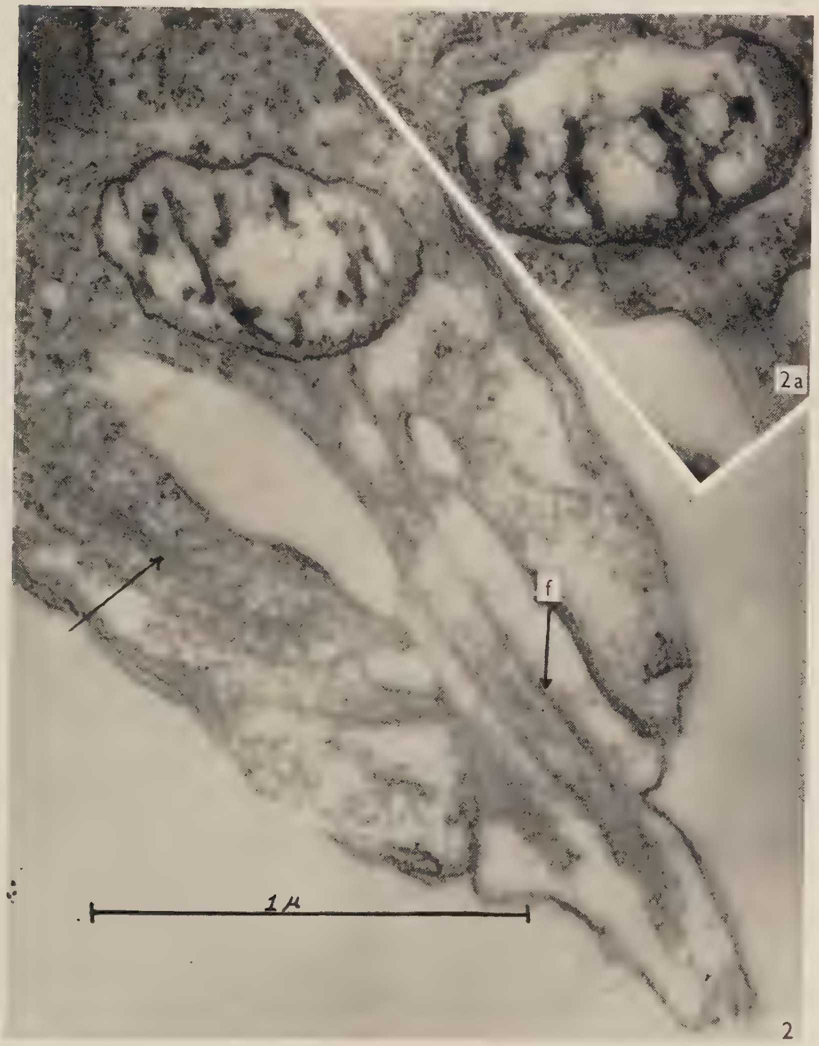
k
1


2a
f
1 μ
2

but a number of sections cut posterior to the point of entry of the flagellum but anterior to the kinetoplast have shown that the two axial fibres may be missing (Fig. 9). While it is possible that the loss of these two fibres may be an artifact of sectioning, similar observations have been made by Rothschild [26], Fawcett and Porter [7] and Wolken and Palade [28] in their studies of cilia and flagella of other types of cells. Afzelius [1] on the other hand claims that in the case of sea urchin spermatozoa the two axial fibres terminate at the same point as the nine peripheral ones. It has not yet been possible to cut sufficient serial sections through a single cell of *S. oncopelti* to determine the distance from the kinetoplast at which the two axial fibres terminate; the longitudinal sections shown in Figs. 2 and 2*a* suggest that these two fibres may extend up to the kinetoplast membrane, but such sections are difficult to interpret accurately since they may be cut at an oblique angle with the kinetoplast uppermost.

The kinetoplast of *Strigomonas oncopelti* appears to consist of two main parts: a central, relatively electron-transparent area and a more electron-dense peripheral region. From the sections examined it seems that the intricate network of filaments and granules in the central zone of the kinetoplast originates from or is a continuation of the nine peripheral fibres of the flagellum, as shown diagrammatically in Text-fig. 2. The width of the peripheral electron-dense area of the kinetoplast appears to depend upon the position of sectioning; little or no peripheral material is found in sections cut through the anterior end of the kinetoplast (Figs. 3 and 5).

Light microscopy of stained preparations of *Strigomonas oncopelti* and numerous other trypanosomid flagellates [2, 4, 14, 16, 25] has shown that the kinetoplast has a Feulgen positive periphery and an achromatic central region; with this in mind it is suggested that the electron-dense peripheral substance seen in thin sections of kinetoplasts may represent the Feulgen positive material of this structure; the similarity in appearance of this sub-

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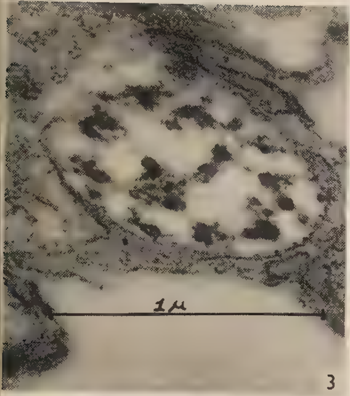
Fig. 3–7.—Transverse sections through kinetoplasts showing the complex network of filaments and granules. Some of these sections (Figs. 4, 6 and 7) show an electron-dense (*ED*) peripheral region which resembles nuclear material (*n*) in appearance.

Fig. 8.—A transverse section through two flagella in one cell showing the typical 9 + 2 arrangement of fibres.

Fig. 9.—A transverse section through two flagella in one cell showing that the two axial fibres of the flagella may be missing.

Figs. 10 and 11.—Transverse sections of flagella showing elongations of the outer sheath to form ribs.



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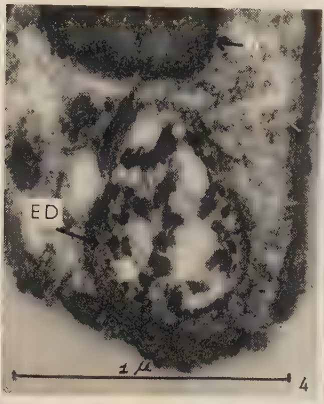

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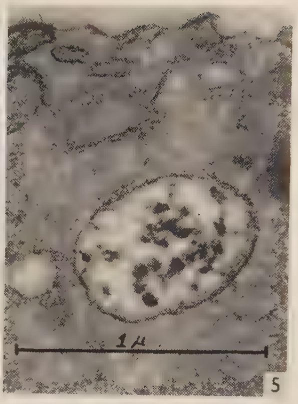

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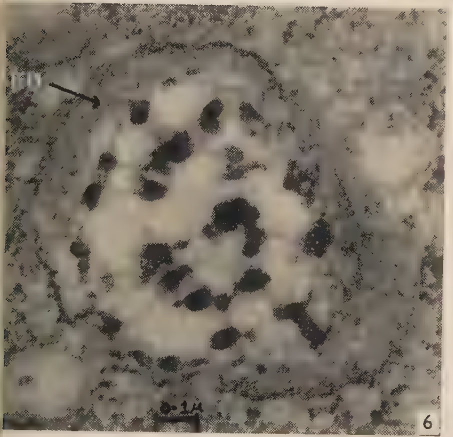

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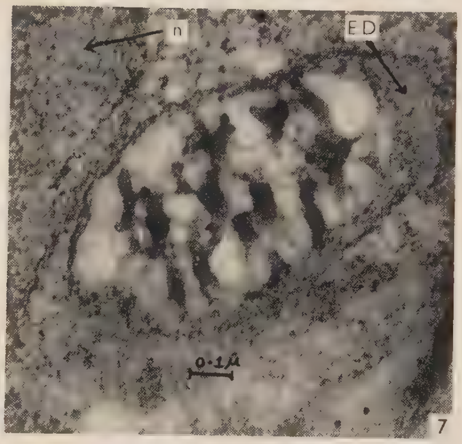

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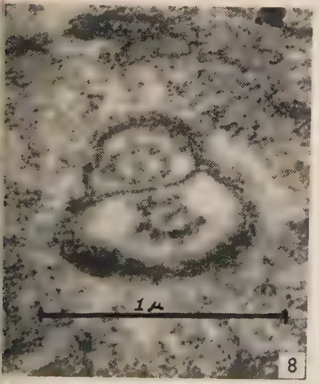

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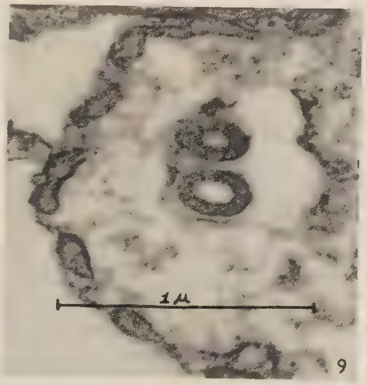

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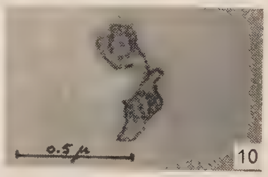

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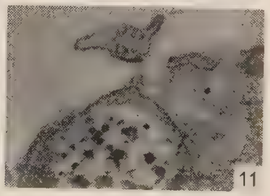

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stance and of the Feulgen positive nuclear material seems to provide some support for this view.

There are insufficient data available at the present time to permit any discussion of the functional significance of the structures described, but it should be pointed out that although the kinetoplast is typically a permanent structure in trypanosomid flagellates, it may be absent in certain individuals in a population, and also akinetoplastic strains can be produced by treatment of flagellates with certain organic dyes [12, 27]. Both induced and spontaneous akinetoplastic strains breed true for an indefinite period and the organisms appear to be normal in every other respect. Hoare [11], discussing the genetic aspects of this phenomenon, suggests that it may be regarded as a mutation determined by plastogenes, the loss of the kinetoplast being comparable to the loss of plastids in phytoflagellates. If the filamentous structure of the kinetoplast is intimately related to the flagellum, as suggested in the present discussion, it seems likely that a loss of the whole kinetoplast structure might result in a loss of motility, but this has not been observed. It seems possible that akinetoplastic organisms may only have lost the peripheral Feulgen positive material of the kinetoplast, the central filamentous structure being present but undetectable by light microscopy. With this hypothesis in mind it is interesting to note that a number of the organic dyes which have been used to produce akinetoplastic strains [23, 27] are acridines or closely related compounds and more recent work [5, 19, 20] has suggested that some of these compounds may be specific inhibitors of deoxyribonucleic acid synthesis. It is hoped that an electron microscope study of akinetoplastic organisms may give some indication of the parts of the kinetoplast–blepharoplast complex which are essential to motility.

SUMMARY

Examination of thin sections of the trypanosomid flagellate *Strigomonas oncopelti* in the electron microscope has revealed some internal structure in the kinetoplast of this organism; two main parts have been distinguished: a central, electron-transparent area which contains a fine network of filaments and granules, and a more electron-dense peripheral area which resembles nuclear material in appearance. In transverse sections of flagella the usual arrangement of 9 peripheral and 2 axial fibres has been observed; some evidence has been obtained which suggests that the two axial fibres may be missing in the region of the kinetoplast. A possible relationship between the flagellum and kinetoplast is discussed.

The authors are indebted to Mr W. Grimstone, of the Zoology Department, Cambridge, for assistance in cutting some of the sections. The electron microscopy was done in the Electron Microscope section of the Cavendish Laboratory, Cambridge, under the supervision of Dr. V. E. Cosslett, with the Siemens "Elmiskop" Electron microscope purchased through the generous benefaction of the Nuffield Foundation. One of us (R. W. H.) is indebted to the Agricultural Research Council for financial aid.

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Experimental Cell Research **15**, *112–117* (*1958*)

NIGROSIN AS A DYE FOR DIFFERENTIATING LIVE AND DEAD[1] ASCITES CELLS

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Received October 24, 1958

In a study related to permeability characteristics of the cell membranes of ascites tumor cells, a dye was sought which would differentiate cells with altered membranes from cells whose semi-permeable membrane was unchanged.

During the last four decades, *in vitro* staining procedures have been used to differentiate between live and dead cells in suspensions, e.g., leucocytes and ascites cells [7, 8, 9, 10]. These methods are based upon the assumption that certain dyes, e.g., trypan blue, safranin, eosin, etc., will stain only dead cells in such a suspension and that the live cells will remain unstained when exposed to these dyes. This ratio of live to dead cells can then be used to quantitate metabolic and cytologic data obtained from cell suspensions exposed to a variety of experimental conditions.

In preliminary studies, it appeared that many dyes had acidic or toxic properties that might be injurious to the live cells in the suspension, and hence could possibly lead to variable results, particularly if the cell suspension had been or was going to be used to obtain metabolic data. The lack of good contrast between the background and the stained or unstained cell was also a disadvantage common to many dyes, and often caused difficulty in differentiating between stained and unstained cells.

In an attempt to find a stain possessing optimal qualities, including ease of visualization, over 50 dyes were screened. Nigrosin, of all the dyes studied, gave the most dramatic and obvious contrast, and was therefore investigated in greater detail.

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[1] This investigation was supported in part by a research fellowship from the National Cancer Institute of the National Institutes of Health, USPHS, and was carried out at the Institute for Cell Research and Genetics, Karolinska Institute, Stockholm, Sweden, and in part by grant C-3152 from the National Cancer Institute, USPHS.

[2] The term "dead cell" will be used in this paper merely as a convenience in nomenclature, since recent evidence indicates that stained cells are not necessarily metabolically dead (see discussion).

It is the purpose of this paper to describe the advantages of nigrosin as a dye for differentiating live and dead cells *in vitro*. A simple method for modifying the permeability of cell membranes of ascites tumor cells is also described. These modified cells were used to test the properties of nigrosin.

EXPERIMENTAL

The nigrosin diluting solution contained 0.2 to 0.5 per cent nigrosin[1] in either isotonic saline or Ringer's medium. Optimal dye concentration depended somewhat on the type of cells to be studied, e.g., tumor cells usually required a stronger dye concentration than leucocytes. Cells to be counted were diluted ten or twenty fold in a diluting pipette (white cell pipette) with the nigrosin solution as the diluent. Cell counts were made in a standard blood counting chamber with Neubauer rulings. Dilutions were made so that at least fifty cells were counted in each of the four large squares of the counting chamber. The results were expressed as cells per milliliter.

Cell suspensions used in these studies were obtained from the ascites form of a MClM methylcholanthrene induced sarcoma [5], and from an Ehrlich ascites carcinoma. The cells were centrifuged from the ascites fluid, and resuspended in isotonic saline or Ringer's medium before diluting for counting.

Experimentally injured cells to test the stain and to study membrane permeability were obtained by subjecting the tumor cells to a hypotonic medium (water) for 1 to 4 minutes. The percentage of modified[2] ascites cells (MA cells) that resulted from this procedure was proportional to the time that the cells were kept in the hypotonic medium. The cells were resuspended in an isotonic medium after the desired ratio of MA cells to unmodified (UM) cells had been obtained (30–50 per cent MA/UM cells in these experiments). Resuspension of cells in an isotonic medium was accomplished by either adding an equal volume of 1.8 per cent NaCl solution, or by centrifuging the cells from the hypotonic medium and resuspending in an isotonic medium.

The speed of modification may be tempered by subjecting the cells to a hypotonic saline solution, e.g., 0.4 per cent NaCl, rather than water. The presence of protein, such as ascites fluid, will inhibit the modification of the cells. Attempts to obtain cell suspensions of 100 per cent modified cells usually resulted in decreased, or loss of, metabolic activity. MA cells and dead cells are stained black by nigrosin, UM cells are unstained.

RESULTS

Data obtained from modified cells are illustrated in the following experiments in order to show that nigrosin had no additional deleterious effect on

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[1] Certified nigrosin, water soluble, National Aniline Division, Allied Chemical & Dye Corporation, New York.

[2] Modified ascites cells is the term applied to cells in which the semipermeable nature of the cell membrane has been changed so that the membrane is permeable, but the cell retains metabolic activity [3]. Modified cells are stained black by nigrosin.

TABLE I. *Modified ascites cells exposed to nigrosin for various periods of time.*

Exp.	Time	Unstained	Stained	Total	% Live
1.	0 mins.	12.9×10^6	10.0×10^6	22.9×10^6	56.3 %
	30 mins.	11.4	8.4	19.8	57.6 %
	150 mins.	11.8	8.8	20.6	57.3 %
2.	0 mins.	13.3×10^6	11.2×10^6	24.5×10^6	54.3 %
	30 mins.	13.6	11.6	25.2	53.9 %
	150 mins.	13.6	9.7	24.3	55.9 %

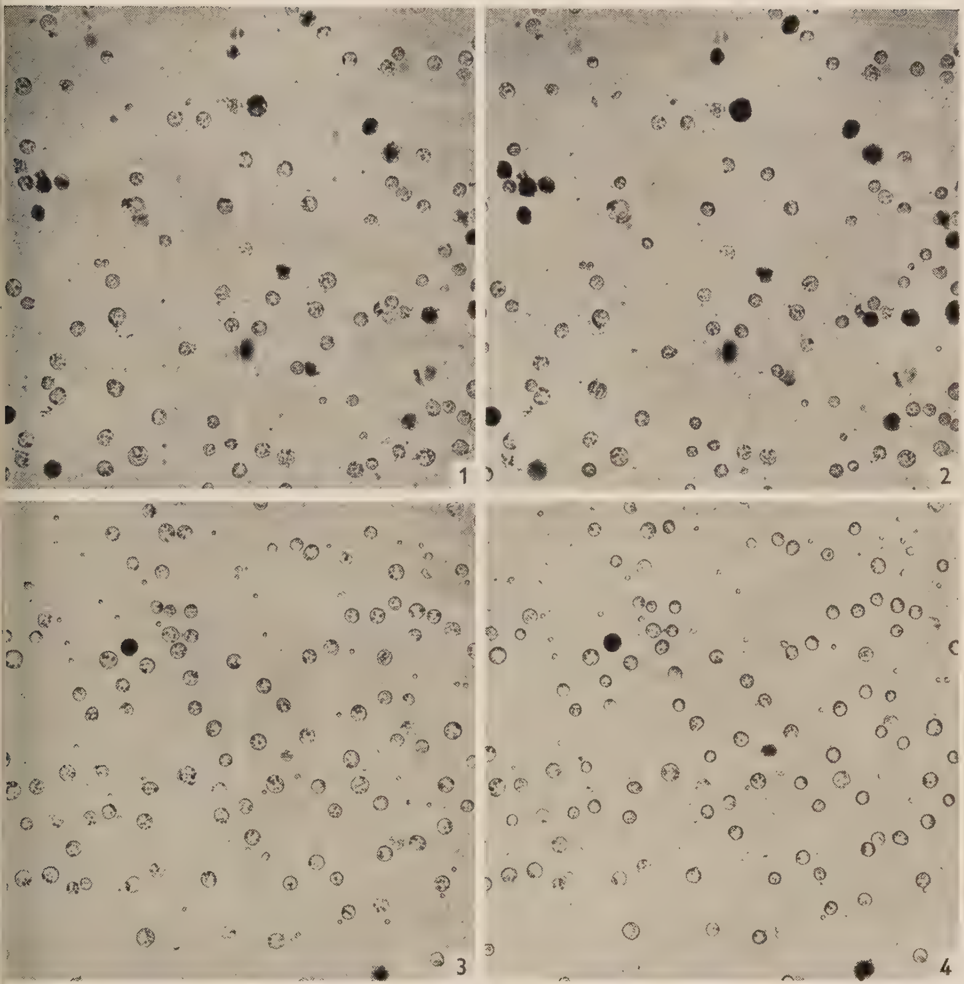
Experiment 1, cells in 3.0 ml nigrosin–Ringer's medium; experiment 2, Same as experiment 1, except 0.5 per cent glucose added. Temperature 22°–26°C.

cells previously exposed to an unphysiological (hypotonic) medium. Similar results were always obtained with unmodified cells.

Results in Table I are representative of a series of experiments demonstrating the non-toxic nature of nigrosin. Cells in Experiment 1 were kept in a nigrosin–Ringer's dilution medium, and cells in Experiment 2 in a nigrosin–Ringer's plus glucose medium. The results indicate that nigrosin had no effect on the number of live cells over a period of 2.5 hours (at room temperature), nor did nigrosin affect the percentage of live cells. In a small number of experiments using unmodified cells, no significant difference in the ratio of stained/unstained cells was observed in a pH range of 6.5 to 7.8. Also, the addition of 1 and 2 mg of nigrosin per 3 ml of Ringer's medium plus glucose containing 10^7 ascites cells had no inhibitory effect on anaerobic glycolysis, as quantitated by lactic acid formation.

The non-toxic nature of nigrosin is further indicated in Figures 1 and 2. Figs. 1 and 2 show modified ascites cells in nigrosin–Ringer's, and kept in a hemacytometer at room temperature for zero and 20 minutes, respectively. Figs. 3 and 4 are the same as Figs. 1 and 2, except that unmodified cells were used. The cells show little or no change in staining or morphological appearance, although the extent of staining increases with time in some of the stained cells.

An additional advantage of nigrosin, demonstrated in Figs. 1–4, is its black color. The black color of the stained cells compared to the relatively light background allows stained cells to be easily photographed and easily visual-



Figs. 1 and 2.—Modified ascites cells in nigrosin–Ringer's for zero and 20 minutes respectively.
Figs. 3 and 4.—Unmodified ascites cells in nigrosin–Ringer's for zero and 20 minutes respectively.

zed upon microscopic examination. Moreover, the background does not obscure the unstained cells.

Nigrosin has also been tested with suspensions of cells from solid tumor, rabbit thymus cells, human leucocytes, and other ascites tumor cells,[1] viz., Krebs-2, Ehrlich-Lettre, Krebs-2 clone D (Hauschka).

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[1] Kindly given by Dr. R. Straube, Argonne Laboratory.

DISCUSSION

Nigrosin belongs to the quinone-imine group of dyes. These dyes are characterized by having two chromophore groups, a quinoid benzene ring and an indamin group. Of the five sub-groups of the quinone-imine dyes, Conn [1] lists nigrosin as an indulin type dye in the azin sub-group. Although a certified dye, nigrosin is a mixture of a blue-black or violet indulin with a yellow dye, resulting in a black color [1].

The black color of nigrosin is easily visualized upon microscopic examination of the stained cells, and is therefore ideally suited to the application of the method of unstained cell counts described by Schrek [9, 10]. Moreover, the apparent non-toxic nature of nigrosin allows a reasonable time to count numerous or replicate samples.

Nigrosin appears to have the greatest affinity for the nuclear and grandular material in the stained cells, although more diffuse staining becomes apparent over a period of time. However, many of the cells are immediately diffusely stained. The extent of cellular staining may be related to the relative intactness of the cell with respect to the semi-permeability of the membrane and the functioning enzyme systems required to maintain cellular integrity.

An earlier study indicated that unmodified ascites cells would utilize glucose alone for glycolysis, whereas modified cell suspensions would utilize glucose in proportion to the number of unstained cells present [4]. More recently, evidence has been presented indicating that stained cells are not necessarily metabolically dead cells, but may have changed in the semi-permeability of the cell membrane resulting in a loss of intracellular co-factors and/or substrates, but not necessarily intracellular enzymes. The addition of glycolytic co-factors [6] to suspensions of modified cells returned the glycolytic activity to the level of unmodified cells [3]. Other metabolic systems and biological functions of MA cells are currently being investigated.

Thus, this staining technique differentiates unchanged cells from those in which the semi-permeability of the cell membrane has been changed, but does not differentiate between cells capable of metabolic function, and cells metabolically dead.

SUMMARY

The application of nigrosin as a stain for differentiating between live cells with unchanged membranes and cells whose membranes have been modified either experimentally or by death of the cell has been described. The advan-

tages of nigrosin have been shown to be its high contrast between stained and unstained cells, and its non-toxic nature.

A simple method is given for modifying ascites cells, i.e., modifying the cell membrane so that it becomes permeable to large molecules such as nigrosin.

The ability of modified cells to metabolize is briefly discussed.

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Experimental Cell Research **15**, *118–131* (*1958*)

AN ELECTRON MICROSCOPIC STUDY OF MITOCHONDRIA FORMATION

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Received October 25, 1957

Mitochondria have been recognized as distinct entities in the cytoplasm since the late nineteenth century. They were first defined as spherical or rod-shaped bodies staining with Altmann's aniline-acid fuchsin stain: the introduction of Janus green staining *in vitro* or *in vivo* provided the first elective method of identification. Their morphology has been described so extensively that we will not attempt here to summarise the classical knowledge and description. More recent, electron microscopic investigations into their morphology have revealed a common pattern in all the recognised types of mitochondria, and further clarified the nature of certain organelles such as the sarcosomes of muscle-fibres, which had been shown to possess certain mitochondrial characters, but proved aberrant by other criteria. In such studies on mitochondrial structure Palade [18] showed that the organelle possesses an outer limiting membrane, composed of two lamellae of about 120–140 A.U. spacing, whilst in the interior there are tranverse double-lamellar membranous partitions which he called "cristae mitochondriales". These he originally thought to be shelf-like prolongations from the inner lamella of the surrounding membrane: more recently it has been recognised that they are more often tubular, finger-like inward projections.

The first attempts to characterise mitochondria chemically were the isolation experiments of Bensley [3] who differentially centrifuged homogenates in 0.9 per cent saline, and separated out organelles which were of the right dimensions but swollen and distorted: chemical analysis of these showed that they have the high lipid content indicated by fixation studies of early morphologists, who found that fixing solutions which were strongly acid or contained organic solvents disintegrated the mitochondria. Morphologically normal mitochondria were isolated by Hogeboom, Claude and Hotchkiss [15] from homogenates prepared in hypertonic (0.88 *M*) sucrose. These isolations led to extensive studies of the enzymatic pattern in mitochondria and it was found that most of the respiratory enzymes of the cell were concentrated in them. This has provided a further criterion for the identification of mitochondrial elements.

The origins and fate of mitochondria in the cytoplasm were not investigated extensively by the earlier cytologists. Various suggestions have been proposed. Some held that they were self-reproducing bodies, dividing extensively at mitosis, others that they were built up from, and could disintegrate into, smaller particles [4]. De Robertis *et al.* [20] have suggested the microsomes as a possible particulate unit from which mitochondria might be built up.

The suggestion that mitochondria might increase in numbers by binary fission has been lent support by recent observations of Fawcett [6] on mitochondria in liver cells of animals suffering inanition. Fawcett obtained convincing pictures of mitochondria undergoing cleavage: he further describes such occurrences occasionally in cells of normal animals. However a good deal of evidence from experimental sources can be marshalled against the suggestion that this phenomenon is widely occurring. Harvey [13] demonstrated, by centrifuging fertilised *Arbacia* eggs, that mitochondria can arise *de novo* in mitochondria-free cytoplasm, whilst the quantitative studies of Gustafsson and Lenicque [11] and Agrell [2] have indicated that increase in numbers of mitochondria in dividing cells occurs only at certain stages of the mitotic cycle, and Agrell further generalises that such increase can occur only in the presence of an intact nuclear membrane.

Hartmann [12] showed that during chromatolysis the mitochondria of the nerve cell increase in numbers, and Causey and Hoffman [5] found evidence suggesting that, in this chromatolytic phase, vesicles were budded off the nuclear membrane, this being later followed by invagination into the interior of the vesicle of crista-like processes, culminating in the formation of typical mitochondria. They further suggested that, with the addition of more cristae, and the accumulation of dense interstitial material between the cristae, the mitochondria eventually transform into typical liposomes. Earlier evidence supporting such a transformation is available in the work of Hirsch [14], and Lever has produced electron microscopic evidence in agreement with the hypothesis [16, 17].

Ben Geren and Schmidt [8] have suggested that mitochondria may be formed from another membrane—the plasma membrane of the giant axon in invertebrates.

In this paper we are concerned with common morphological features, suggestive of mitochondrion formation from the nuclear membrane, encountered in a variety of animal and plant cells. Three main cell types have been studied: rat thymus lymphocytes, from animals ranging in age from newborn to adult; adult mouse lymph node, spermatogonia and spermatocytes of rat testis, and meristematic cells of onion root tip.

MATERIAL AND METHODS

The tissues were all fixed in Palade's 1 per cent osmium tetroxide solution, buffered at 7.2 to 7.4 pH, at approximately × 4°C, the method of application varying with the tissue. Onion root tips were amputated and dropped into chilled fixing solution, rat thymus and mouse lymph nodes were bathed briefly with the fixing solution *in situ*, then small fragments were dissected into chilled solution, while testis tubules were exposed to the fixing solution by incising the capsule and pouring fluid over extruded material. Fixation of testis and root tip was found to be optimal after 2 hours, while thymus and lymph node required no more than one hour.

After a five-minute rinse in distilled water, the tissues were dehydrated automatically in a tissue dehydrator [9] resulting in their transfer to absolute alcohol in approximately one hour. They were then transferred into 50 per cent alcohol, 50 per cent ether for about ten minutes, and infiltrated with 4 per cent histological celloidin at 60°C under pressure for 8–12 hours. After cooling, the tissue fragments were set in celloidin blocks by treatment with chloroform, trimmed to cubes of about 1 mm side, and transferred to gelatin capsules containing a methacrylate monomer mixture with catalyst (15 per cent methyl methacrylate, 85 per cent butyl methacrylate, with addition of 2 per cent benzoyl peroxide) and the contents polymerised by ultraviolet light. This double imbedding technique is described extensively elsewhere [9].

Sections were cut on a thermal expansion ultramicrotome, transferred to copper grids coated with formvar films, and when dry were treated with carbon tetrachloride to remove the methacrylate polymer, leaving a celloidin basis within the sections.

Sections were examined and photographed in a Philips E.M. 100 microscope,[1] some being re-examined in a Siemens U.M. 100_{e4},[2] for instance Fig. 7.

OBSERVATIONS

The cells on which these studies have been made differ somewhat in character, but are similar in that all are undergoing frequent division, and show basic similarities in appearance of nucleus and mitochondria.

As may be seen in Fig. 1, the mitochondria are typical in appearance, possessing distinct double-lamella cristae, many of which are of tubular

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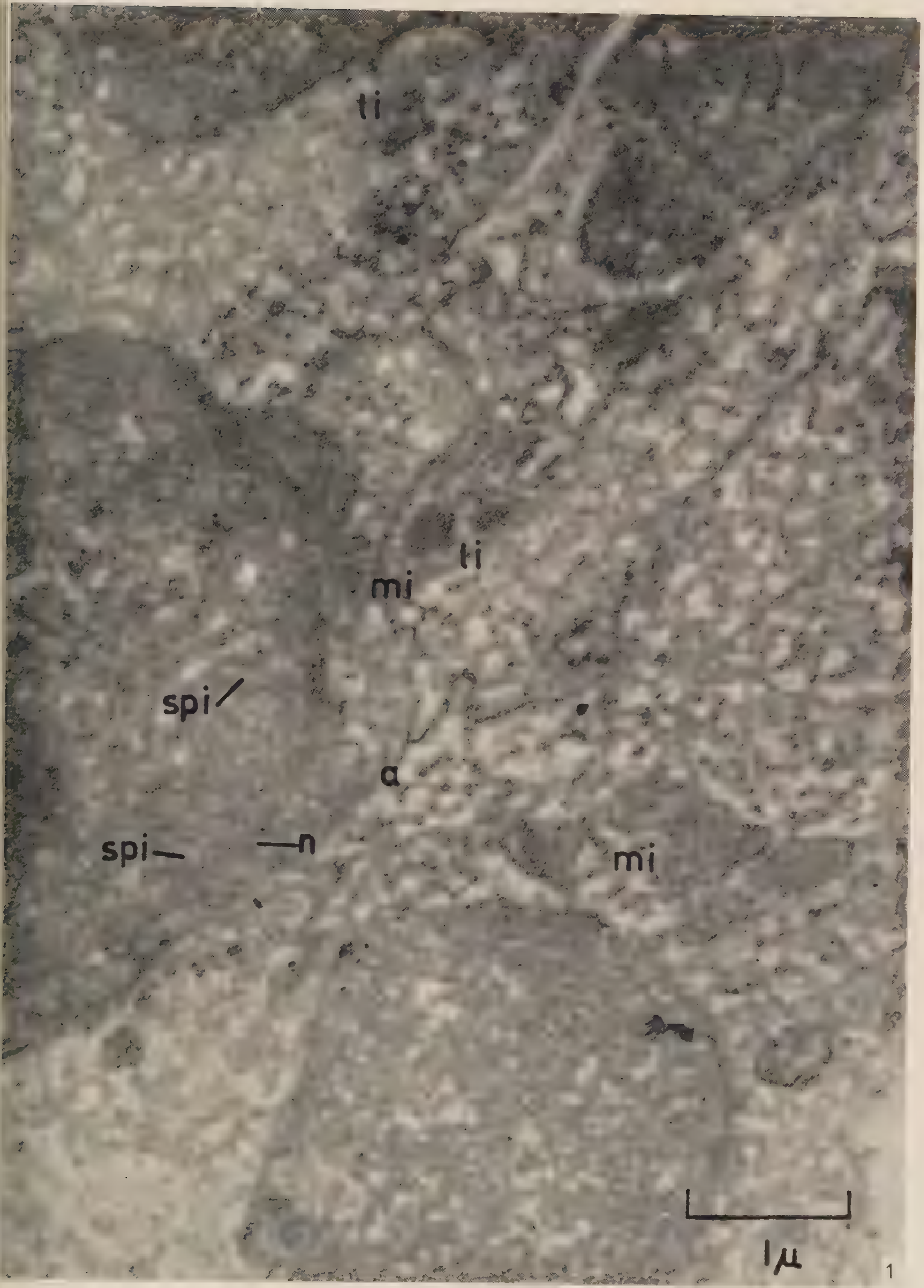
[1] By courtesy Dr. S. Tomlin.

[2] By courtesy Sir MacFarlane Burnet.

Fig. 1.—A group of typical large lymphocytes from thymus of 6-day-old rat. Typical mitochondria may be seen (*mi*) as well as an incompletely developed one (*a*) and concentrically lamellated liposomes (*li*). Within the nucleus masses of spiralised filaments may be seen (*spi*).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form. Between the cristae traces of an interstitial material may be detected. In Fig. 1 a few concentric-lamellated liposomes may also be observed.

The cell nucleus is usually oval or round in section, and smooth in outline, a shape suggestive of turgidity. It is bounded by a complex double-lamella membrane, clearly seen in Figs. 1 and 8, lamellae being spaced at 100–200 AU and frequently showing the pore structure described by Afzelius [1] and Watson [20]. The interior of the nucleus is largely occupied by tangled spiralised filaments, composite structures containing desoxyribonucleic acid and protein [10] approximately 50–70 A.U. in diameter, well illustrated in Fig. 1. The extremely pleomorphic, but usually denser nucleolus appears to be composed of dense but amorphous material superimposed on a fibrillar chromosomal substratum. The spiralised nucleoprotein filaments appear to have no constant relation to the nuclear membrane.

In actively dividing cells, a proportion of nuclei are highly convoluted, showing many pseudopodial processes, as in Fig. 2, representing rat thymus lymphocytes. Around the concavities in the nuclear membrane mitochondria are often clustered, as seen in Figs. 2, 6, 8, 12 and 13, some lying in very close juxtaposition to the membrane.

Connections between nuclear membrane and mitochondria limiting membrane are often seen: these may consist of short, broad stems such as in Figs. 3, 9, and 14, or long tenuous tubular strands as seen in Figs. 7, 8 and 12. These closely apposed, connected mitochondria can be identified in living material stained supravitally with Janus Black, or viewed by phase contrast

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Fig. 2.—A number of cells from adult rat thymus, at moderate magnification. Four nuclei are distinctly pseudopodial in appearance, and mitochondria are in close association with nuclear membranes at various points (*arrows*).

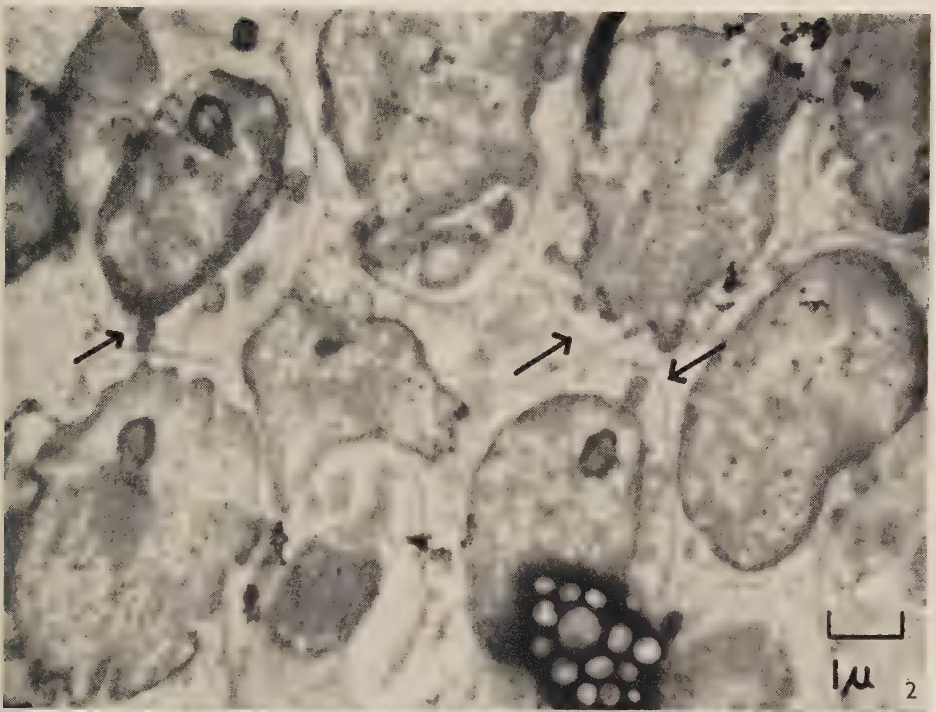
Fig. 3.—In this picture we see portions of two cells; in the left cell, at *c*, a mitochondrion still connected with the nuclear membrane may be observed: apart from this connection it appears typical. At *d*, on the nuclear membrane of the cell on the right, the situation is rather confused, but one mitochondrion, broadly connected with the nuclear membrane, is connected by a narrow neck with a second mitochondrion. (6-day-old rat thymus.)

Fig. 4.—Just below the nuclear membrane may be seen a large intranuclear mitochondrion (*inm*). Crista-like structures of double-membrane are indicated (*c*). (From lymph node of adult mouse.)

Fig. 5.—In this figure another intranuclear organelle is shown (*inm*), just below the nuclear membrane: though denser than that seen in the previous figure it is enclosed by a double membrane, and contains recognisable cristae of double lamella (*c*). (Mouse lymph node, adult.)

Fig. 6.—This figure illustrates a mitochondrion connected with the nuclear membrane by only a narrow neck of outer lamella (arrow). (6-day-old rat thymus.)

Fig. 7.—Another fully developed mitochondrion connected with the nuclear membrane by only a narrow neck (arrow). (6-day-old rat thymus.)



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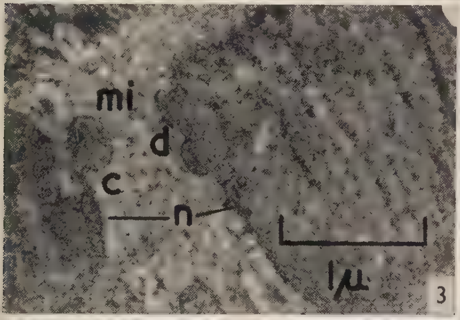

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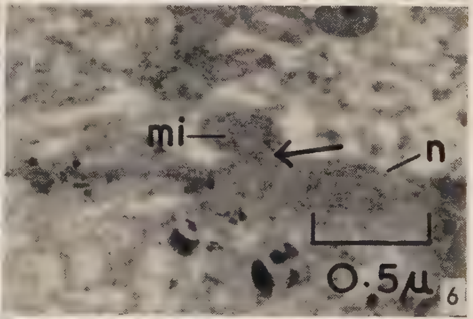

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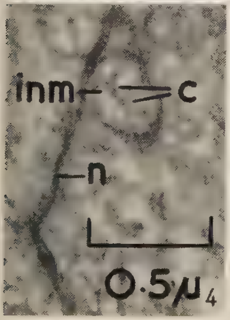

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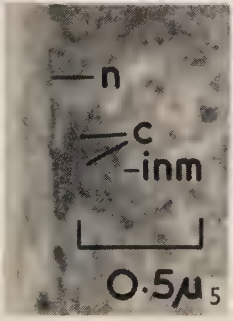

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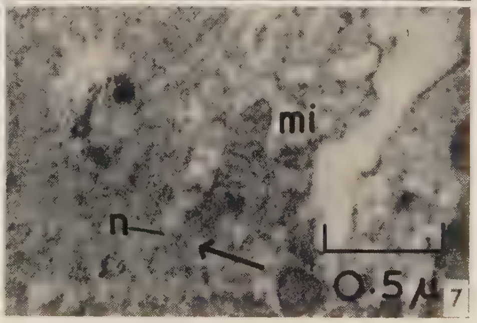

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methods, and although their connections with the nucleus cannot be seen, their association with the nuclear surface is obvious.

The attached organelles possess a double limiting membrane, usually continuous in both lamellae with that of the nucleus, as in Figs. 3, 9 and 14, and commonly they posses typical cristae of transverse sheet or tubular type. Occasionally only a few short cristae are present, but even these are usually identifiable. In some cases only the outer lamella of the mitochondrial and the nuclear membrane are continuous, the inner lamellae of each being separate and intact: this may be seen in Figs. 6, 7, and 11.

In many instances where direct continuity with the nuclear membrane cannot be demonstrated, mitochondria with long (double-layered) strands emerging from their outer membrane lamella may be observed, as in Fig. 8. Here such a mitochondrion lies close to another whose connection with the nuclear membrane is obvious. The nuclear membrane often possesses tubular processes which project into the cytoplasm, as seen in Fig. 8. These projections, illustrated earlier by Causey and Hoffman [5] are often extremely elongated and although their connections with mitochondria cannot always be established, it seems probable that they are fragments of nucleo-mitochondrial connection.

Attached mitochondria are frequently found adjacent to concavities in the nucleus, of roughly similar diameter to the mitochondrion. Such concavities may sometimes be partly roofed by projections from the nuclear membrane on the side opposite to the attached mitochondrion, as illustrated in Fig. 14. This observation might suggest that the mitochondrion is formed within the nucleus and extruded with a transient stem-like connection to the nuclear membrane. In some tissues, root tip and nerve cell [5] there is frequently a clump of dense granular material close to the base of the attached mitochondrion.

Within the nucleus there are frequently appearances suggestive of synthetic activity by the membrane. Just beneath it there are often regions differing markedly in appearance from the rest of the interior: they are commonly less dense, lack spiralised filaments, and are separated off by a layer of double-lamellated membrane similar in spacing to the nuclear membrane. These membranes, usually semi-circular in section are continuous with the

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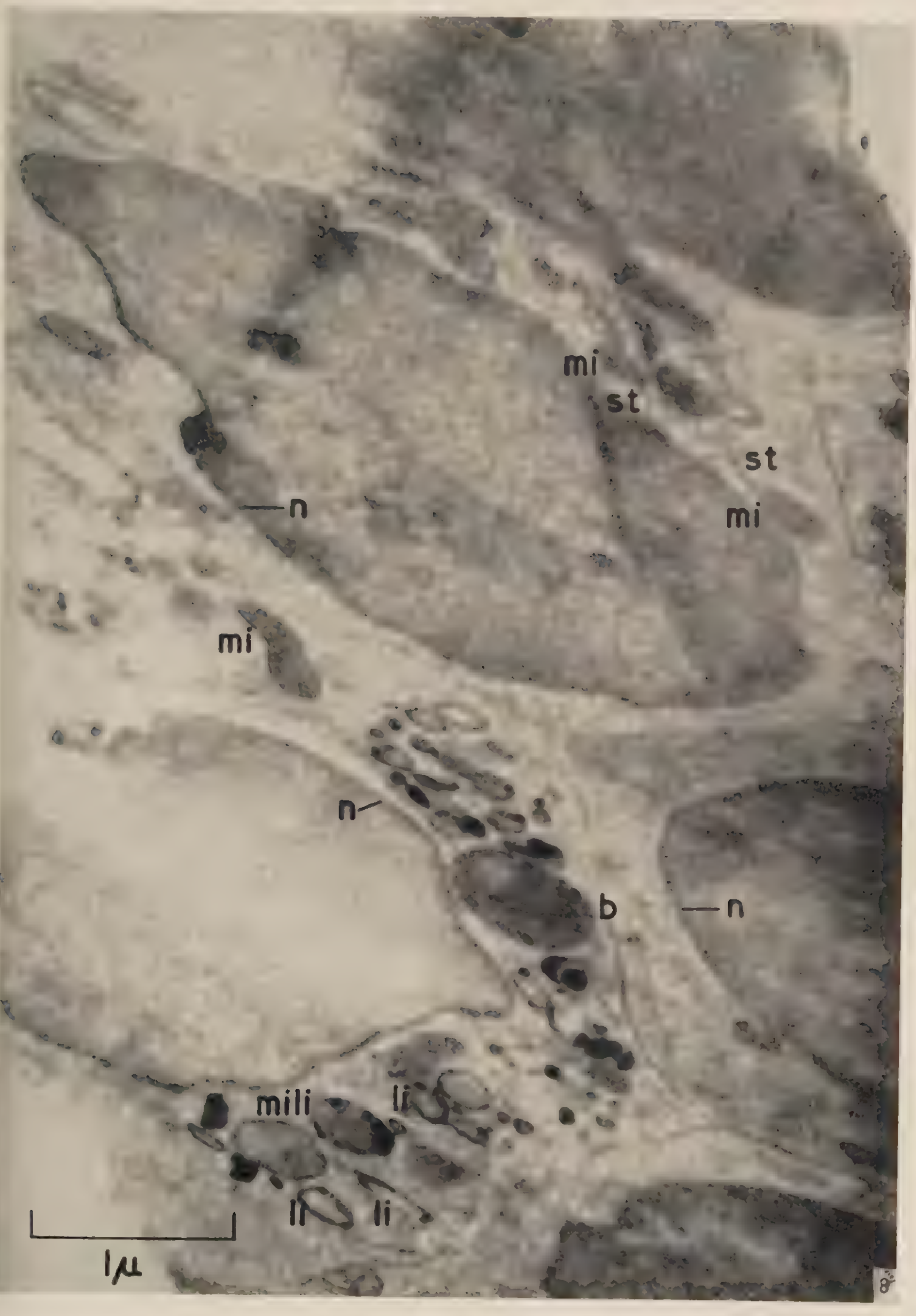
Fig. 8.—A group of large lymphocytes from 6-day-old rat thymus. The cell in the upper part of the field has one mitochondrion clearly attached to the nuclear membrane, next to a concavity, by a long, narrow stem (*st*), while two other mitochondria near it also have elongate extensions, though not reaching the nucleus.



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nuclear membrane at one end, often also at the other. Membrane enclosed areas of this type may be seen in Figs. 4, 5 and 16 where the membrane is well defined, and in Figs, 9, 12, 13 and 18, where, although the electron density of the nucleus is higher, the intranuclear membrane is recognisable. Within this enclosed area membranous elements, reminiscent of cristae mitochondriales may be detected, as in Figs. 4, 13 and 18. Both these and the limiting membranes tend to be rather less dense than those of extra-nuclear mitochondria.

In some regions of the nucleus numerous villiform inward projections can be seen, originating from the inner lamella of the nuclear membrane: these, examples of which can be seen in Figs. 10, 13 and 15 are of approximately the same spacing as the parent membrane, often closely packed, and range in length from 0.1 to 0.5 micron.

Liposomal bodies of characteristic appearance may be seen in Figs. 1 and 8, including forms which appear transitional between typical mitochondria and tightly lamellated lipid bodies. Such structures as seen in Fig. 1 contain lamellar elements similar to mitochondrial cristae, but in Fig. 8 a number of rather atypical organelles may be seen, suggestive of mitochondria within which dense spherical bodies are being elaborated. Possibly this is another mode of transformation to liposomes.

The cell in the lower left possesses a variety of organelles: typical mitochondria may be observed (*mi*) as well as one very large structure with internal membranes *b*, several transitional mitochondria-like elements and bodies with varying numbers of dense objects inside them *li*. In one instance dense bodies occur within an otherwise typical mitochondrion (*mi*, *li*).

Fig. 9.—In this figure, from 6-day-old rat thymus, the mitochondrion at *cmi* appears related to the nuclear membrane by a lamellar structure. Just below the membrane at this point, within the nucleus there is a differentiated region, *inm 1*, delimited by membrane lamellae, within which crista-like circles may be seen. Another such region can be seen at *inm 2*, possessing similar crista-like elements.

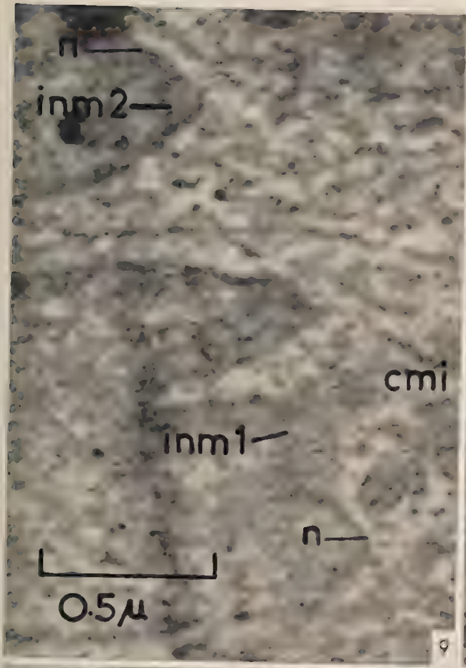
Fig. 10.—This figure is from the same material as Fig. 9: it shows villous elements (*v*) projecting inward into the nucleus from the nuclear membrane.

Fig. 11.—A connected mitochondrion, from onion root meristematic cell. In this instance only the outer lamella of the mitochondria membrane is continuous with that of the nuclear membrane.

Fig. 12.—The entire lower pole of the nucleus occupying the upper half of the field is rich in membranous elements e.g. at *inm 1, 2*. Particularly at *inm 2* a region, enclosed by membrane, appears to be packed with vesicles. (6-day-old rat thymus.)

Fig. 13.—Intranuclear structures are shown at *inm:* clearly defined by double-lamella membranes, they possess internal crista-like elements. Villi originating from the nuclear membrane may be seen at *v*. (6-day-old rat thymus.)

Fig. 14.—A connected mitochondrion from onion root meristematic cell: this organelle lies alongside a concavity in the nucleus, from which it may have emerged.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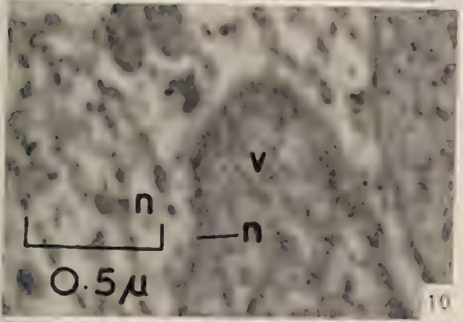

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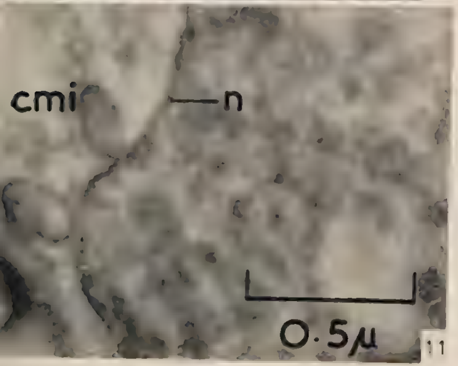

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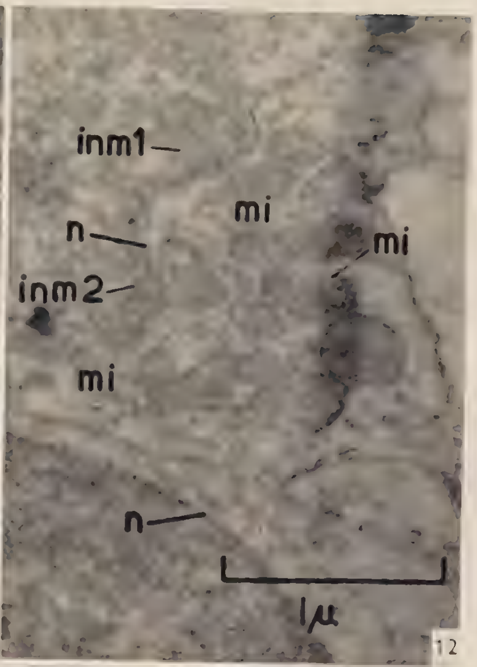
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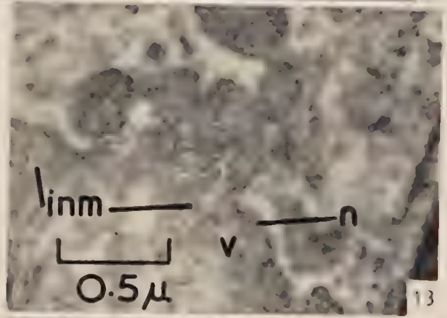

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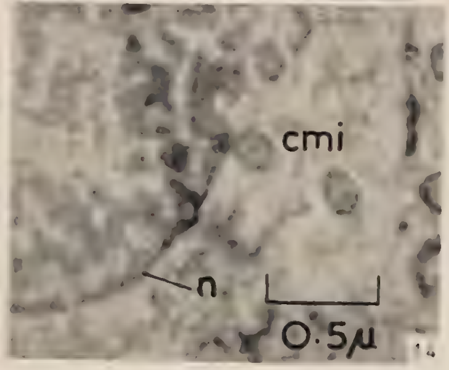

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DISCUSSION

Although there are minor differences in the mode of mitochondrial formation in the various tissues studied, there is considerable basic similarity in the observed phenomena in the most widely differing animal and plant cells. This lends further support to the suggestion that the mechanism of formation described here is a general one.

The evidence presented above leads us to suggest that the membranous system which is described as the mitochondrion is elaborated within the nucleus, primarily by extensions from the inner lamella of the nuclear membrane. It may be that the cristae observed within these intranuclear differentiated regions are derived from the villiform projections found so plentifully on the nuclear membrane. The rather lower electron density of the membranes of these intranuclear organelles, in comparison with the typical mitochondrial membranes, may be due to absence or incomplete character of enzymatic and other proteins in the membrane skeleton.

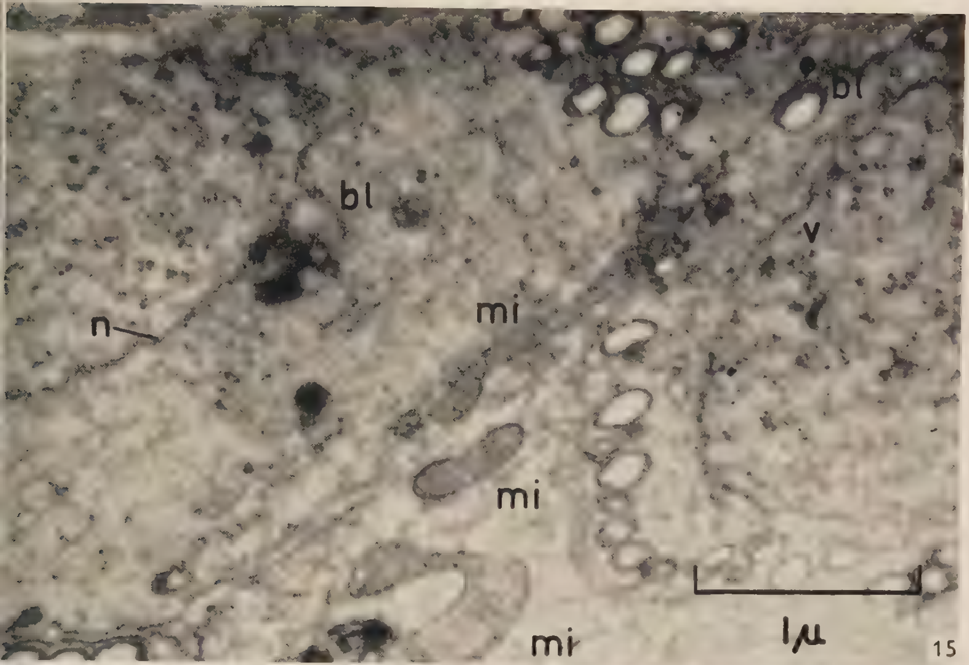
The stages between elaboration of the intranuclear membranous skeleton and the extrusion of a recognisable mitochondrion are by no means clear at this stage—in fact earlier work [5] failed to recognise the intranuclear stages. However, in some manner the mitochondrion appear to extrude first one side, then symmetrically on both sides, sometimes leaving a crater-like cavity alongside (Fig. 14). The occurrence of this concavity in the nuclear membrane leads us to suggest that possibly part of the nuclear membrane ruptures when the mitochondrion is extruded. It seems possible that the nuclear membrane is renewed beneath the emerging mitochondrion, but no evidence has been found in support of this suggestion. Having erupted from the parent nucleus, the organelle at first remains attached by a broad stem, which is gradually stretched, becoming longer and thinner, and persisting eventually over considerable distances.

Fig. 15.—Two spermatocytes from rat testis: the nucleus on the left shows numerous inward-projecting villi (*v*), while the nucleus on the right shows numerous blebs (*bl*) on the outer lamella of the membrane. The vacuolated mitochondria are a characteristic feature of this tissue.

Fig. 16.—Two semicircular invaginations from the nuclear membrane (*inv*) can be seen in this figure. Outside the nucleus is a very closely associated mitochondrion (*mi*). (Spermatocyte, rat testis.)

Fig. 17.—This represents a higher-powered view of the intra-nuclear villi seen in Fig. 14. They appear to be invaginations from the inner lamella of the membrane, but occasionally they also project into the cytoplasm.

Fig. 18.—Just beneath the nuclear membrane, close to the region opposite the mitochondrion is a double-membrane-enclosed region, containing crista-like forms. (6-day-old rat thymus.)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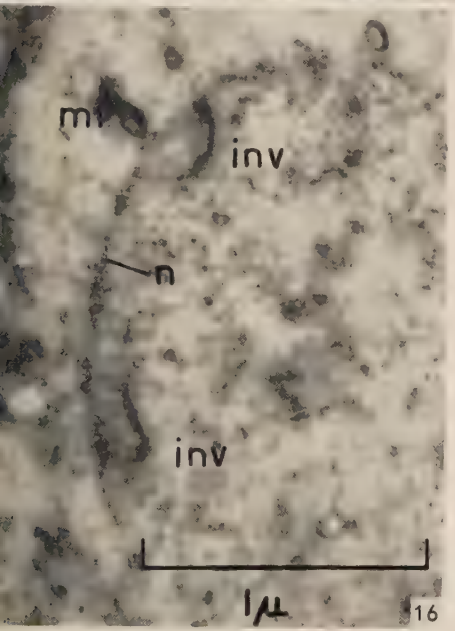
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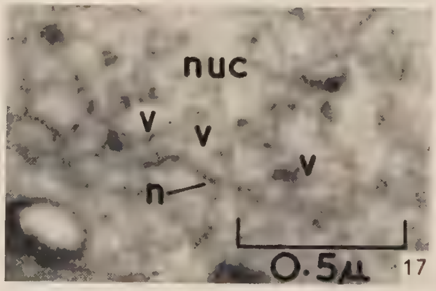
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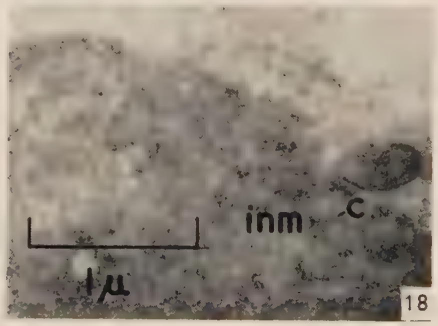

18

The intranuclear membranes begin to form in a characteristic way: growth commences at some point on the nuclear membrane, curving round to meet the parent membrane again one or two microns away. There may be a persistent aperture in this intranuclear membrane, through which continuity between nucleus and mitochondrion are maintained, and this may be the origin of continuity of contents through the stem of the extranuclear mitochondrion, however such continuity is not a constant feature: often only the outer lamella of each membrane is connected.

A significant feature of the evidence presented above, and of deviation from the earlier work of Causey and Hoffman is that the mitochondrion leaves the nucleus fully formed as regards the membranous skeleton. The suggestion that hollow vesicles were budded off, within which, after detachment, invagination proceeded to form cristae, seems to have been due to the well known difficulty of fixing nerve-cells satisfactorily.

Our observations that mitochondria are formed in and extruded from the nucleus do not, of course, exclude other modes of formation such as the binary fission which Fawcett [6] observed in liver cells during inanition. Nevertheless Harvey's [13] experimental evidence for *de novo* origin of mitochondria, and the quantitative studies of Gustafsson and Lenicque, and Agrell, support the hypothesis of nuclear origin.

The extrusion of mitochondria from the nucleus may represent a similar phenomenon to the nuclear projections described by Gay [1] and Lever [16, 17] and such projections have been observed in our material alongside mitochondrial extrusion: the relationship cannot be determined here, however.

The evidence which we have obtained in regard to nuclear origin of mitochondria leads us to suggest that it is the main mode of formation. The case of the giant invertebrate axons raised by Ben Geren and Schmidt [8] must be considered a special one: the volume of cytoplasm served by one nucleus is exceptionally large, and presumably the nucleus is inadequate as a mitochondrial source in these circumstances, when the plasma membrane has taken over the function. We have never observed mitochondria attached to the plasma membrane in other sites.

Since the mitochondria formation which we have studied is occurring in cells undergoing frequent mitosis, it would appear that mitochondria formation is one of the important activities in the interphase nucleus: Agrell comments that increase in mitochondrial numbers occurs only in the presence of an intact nuclear membrane.

SUMMARY

In actively dividing cells mitochondria are commonly found to be attached o the nuclear membrane; the nuclear outline is often pseudopodial in such ituations. Examination of the interior of the nucleus reveals specialised egions demarcated by invaginations of nuclear membrane, which are uggestive of developing mitochondria. On this basis it is suggested that nitochondria are formed within the nucleus from nuclear membrane, and ater extruded into the cytoplasm.

It is suggested that in actively dividing cells this is the principal mode of nitochondria formation: other suggested mechanisms are not, however, xcluded.

We are indebted to Professor J. H. Bennett for his hospitality and to Dr. S. Tomlin nd Sir MacFarlane Burnet for the use of the Phillips EM 100 and the Siemens U.M. 00$_{e1}$ electron microscopes.

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MITOTIC AND CYTOLOGICAL ALTERATIONS OF FIBROBLASTS *IN VITRO* UNDER THE ACTION OF ETHIONINE AND METHIONINE[1]

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Received October 25, 1957

Dyer [4] demonstrated that ethionine (α-amino-γ-ethyl-thiobutyric acid) hinders the growth of animals on a cystine-deficient diet. This effect could be reversed by methionine administration. Other authors studied this toxic effect and tried to elucidate its mechanism [25, 9, 10, 7, 23, 29]. Levine and Tarver [16], Gross and Tarver [11] and Rabinovitz *et al.* [18] showed that ethionine under several conditions is to some extent incorporated into proteins.

Francis and Winnick [8] studied biochemically the action of ethionine on protein synthesis in tissue culture. They demonstrated that this amino acid analogue inhibited the uptake of labelled methionine by proteins and nucleic acid synthesis in heart tissue cultures. Methionine at levels higher than 6.702×10^{-3} M damaged the cultures, particularly when the DL-form was used.

In this paper some cytological aspects of tissue cultures exposed to the action of ethionine and methionine have been studied.

MATERIAL AND METHODS

Hanging drop cultures from the heart tissue of 8-day Leghorn chick embryos were studied. Plasma and 8-day chick embryo extract diluted 1:5 with Tyrode's solution were used in equal parts. On the 2nd day of incubation at 37°C, after washing twice in Tyrode's solution, either methionine or ethionine were added to the fresh medium to a final concentration of 0.03 M and 0.016 M. When both methionine and ethionine were added, each was used at 0.015 M or 0.008 M concentration. In the control cultures, these drugs were omitted. The total number of cultures was 427, 61 being studied for each treatment. After 1, 4 and 48 hours, some cultures were fixed with Carnoy and stained with gallocyanin-chromalum according to Einarson [5] and others studied under a phase contrast microscope.

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[1] Aided by grants of the Conselho Nacional de Pesquisas and Rockefeller Foundation.

[2] Fellow of the Conselho Nacional de Pesquisas.

Some of the cultures, after exposure to the drugs, were washed with Tyrode's olution and incubated with fresh nutrient medium (with or without methionine dded at a final concentration of 0.016 *M*) for further 48 hours.

A few experiments were started with heart explants previously exposed for 1 and 4 ours at 37°C to 0.03 *M* of either ethionine, methionine or both dissolved in Tyrode's olution. Afterwards, the explants were washed three times in Tyrode's solution and ultured the usual way. The controls explants were treated with Tyrode's solution nly and these cultures were studied 48 hours later.

Mitotic counts were performed on gallocyanin-chromalum stained cultures.

RESULTS

Ethionine at a concentration of 0.016 *M* produced definite effects on the n *vitro* grown fibroblasts after 48 hours. The most evident were retraction f cell processes, vacuolation and fragmentation of the cytoplasm, rounding nd shortening of the mitochondria, production of irregular contour of the ucleus and nucleolus, decrease of cytoplasmic basophilia and pycnosis Fig. 1).

The same phenomena were shown to occur as early as 24 hours after xposure to the drug at a concentration of 0.03 *M* of ethionine.

Methionine, when present in the medium at the same concentration as the thionine, completely inhibited the effects of the latter at the lower dosage evels (Fig. 2); this inhibition was however partial at the higher dosage level. ultures grown in the presence of methionine at concentration of 0.016 *M* vere not distinguishable from the controls (Fig. 3). On the other hand, .03 *M* methionine produced effects similar to those of 0.016 *M* ethionine, lthough less severe (Fig. 4).

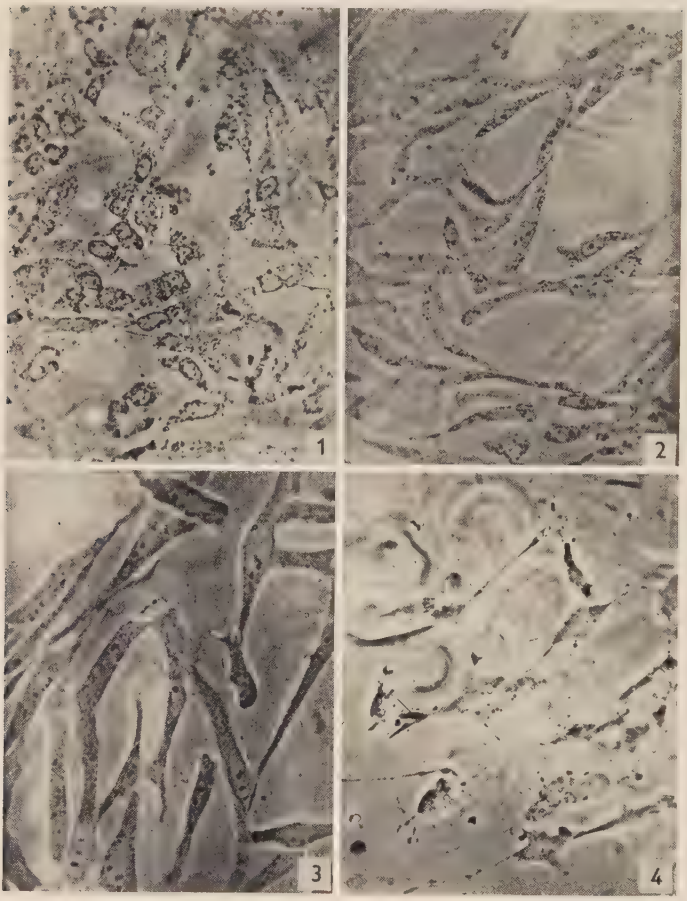
It was also observed that the cytologic phenomena were not reversible y washing and supplying the cultures with normal nutrient medium or vith medium containing 0.016 *M* methionine.

The cultures exposed at the time of explantation to 0.03 *M* methionine for hours at 37°C showed comparatively bad growth, although they could ot be distinguished from the controls by their cytological aspects.

The results of mitotic counts of cultures with the amino acids added at .03 *M* dosage and their controls are presented in Table I.

For each period of exposure to the drugs, tests of homogeneity by the χ^2 ethod were made for the total mitotic counts and the four treatments. or each period the χ^2 obtained were highly significant ($P < 0.001$) suggesting on homogeneity due to the effect of the different treatments.

The global effect of methionine, studied by testing the groups methionine +



Figs. 1–4.—Eight-day chick embryo heart explants cultivated for 2 days, washed and grown on medium containing 0.016 *M* ethionine (Fig. 1), ethionine and methionine 0.008 *M* (Fig. 2), control medium (Fig. 3), methionine 0.03 *M* (Fig. 4) for further 2 days. The cultures were photographed unfixed under the phase microscope. Contrast normal appearance of fibroblasts in Figs. 2 and 3, with the slightly altered cells in Fig. 1 (see text).

ethionine-methionine against the control + ethionine groups was not found to be significant ($P > 0.05$) for the three periods studied.

The global effect of ethionine was not significant ($P > 0.10 < 0.25$) for the two days group, but significant ($P < 0.005$) for the one and four hours groups.

The interaction methionine-ethionine was found to be highly significant ($P < 0.001$) for the three periods studied. It was also found that methionine,

TABLE I. *Effect of methionine, ethionine or both on the mitotic activity of chick fibroblasts grown* in vitro.[a]

Period of exposure to the drugs[b]	Mitotic phases	Control		Ethionine		Methionine		Ethionine + Methionine	
		Mitosis found	Mitosis per 10,000 cells	Mitosis found	Mitosis per 10,000 cells	Mitosis found	Mitosis per 10,000 cells	Mitosis found	Mitosis per 10,000 cells
1 hour	Prophase	16	77.74	11	23.78	3	32.67	17	41.74
	Metaphase	3	14.57	0	0	1	10.89	8	19.64
	Anaphase	4	19.43	1	2.16	0	0	5	12.27
	Telophase	4	19.43	1	2.16	1	10.89	3	7.36
Actual number of counted cells		2,058		4,625		918		4,072	
4 hours	Prophase	49	71.08	36	23.50	49	49.25	32	28.72
	Metaphase	41	59.48	21	13.71	37	37.19	32	28.72
	Anaphase	16	23.21	10	6.52	15	15.07	14	12.56
	Telophase	20	29.01	14	9.14	16	16.08	21	18.84
Actual number of counted cells		6,893		15,315		9,948		11,141	
2 days	Prophase	6	33.59	0	0	16	15.92	5	20.44
	Metaphase	3	16.79	0	0	6	5.97	4	16.35
	Anaphase	7	39.19	0	0	7	6.96	3	12.26
	Telophase	1	5.59	1	4.27	4	3.98	1	4.08
Actual number of counted cells		1,786		2,339		10,044		2,445	

[a] For each treatment the first column gives the actual number of mitosis and total number of cells counted, and, the second, the number of mitosis in 10,000 cells.

[b] Explants grown for 48 hours, washed and afterwards exposed to the nutrient containing 0.03 *M* of the drugs and grown for further 48 hours preceding fixation.

ethionine and ethionine-methionine significantly reduced the number of mitosis in the three experiments when compared to the controls.

The frequencies of the various mitotic stages was studied for the four hours group. A non significant χ^2 ($P > 0.9$) was obtained, showing that the hypothesis that the frequencies of the different stages are the same for the various treatments cannot be rejected.

DISCUSSION

We have shown that ethionine induces cytological alterations in fibroblasts grown *in vitro* and also have observed a pronounced mitosis-inhibiting effect. It can be seen too that mitotic counts were influenced by ethionine before any cytological effects were apparent. The finding that the proportions of the mitotic phases were not modified by ethionine suggests that the pre-prophase period of the cell life is sensitive to the drug. These toxic or inhibitory actions of ethionine at 0.016 M concentration could be prevented by the simultaneous administration of methionine. This fact is similar to that observed with other amino acids analogues [1, 17]. It was also found that, once started, the cytological alterations could not be reversed through the substitution of the nutritive medium to which methionine was added.

Several mechanisms have been suggested to explain these and other phenomena induced by ethionine and their prevention by methionine. Some authors are of the opinion that ethionine prevents the incorporation of various amino acids into the protein structure [6, 24, 8, 20]; others believe the analogue of methionine to impede the demethylation [6, 23, 24], and according to others it could have a toxic action on several enzymatic systems [2, 12, 29, 14]. There are also indications that ethionine may take part in the intermediary metabolism [26, 27, 28, 3, 21, 15, 22] and so form an abnormal protein [30, 16, 11, 19]; ethionine, once incorporated, would not be displaced by its natural homologue [11, 18]. Since the action of ethionine seems to be related to methionine metabolism, an inhibition of purine and thymine synthesis could occur because methionine seems to be involved as a C_1 donor for the synthesis of these compounds [13].

SUMMARY

The action of ethionine and methionine on fibroblasts of heart explants grown *in vitro* was studied. It was observed that ethionine inhibits mitosis in addition to producing cytological modifications. Pre-prophase seems to be the phase most affected by the drug. Methionine showed an antagonistic effect when administered simultaneously with its homologue.

The mechanism of action of these amino acids is discussed.

The authors wish to express their thanks to Prof. J. W. Fertig, Prof. L. C. U. Junqueira and Dr. M. Rabinovitch for many helpful suggestions.

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SH COMPOUNDS IN MITOSIS

II. THE EFFECT OF MERCAPTOETHANOL ON THE STRUCTURE OF THE MITOTIC APPARATUS IN SEA URCHIN EGGS[1]

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Earlier findings concerning the role of thiol groups in the assembly of spindles and asters [4] predicted that the introduction of a high concentration of –SH into a dividing cell should interfere with the formation of the mitotic apparatus. This prediction was confirmed by a recently published study [5] of the effect of mercaptoethanol (monothioethylene glycol) on mitosis in the eggs of the sand dollar *Dendraster excentricus*. While the eggs of *Dendraster* are very well suited for the exploratory study because of the splendid visibility of the mitotic apparatus in the living cell, they are not obtainable in sufficiently large amounts to permit easy isolation of the mitotic figures according to the methods developed in this laboratory [4]. The following studies of mitotic blockage by mercaptoethanol in the egg of the sea urchin *Strongylocentrotus purpuratus* center on the following questions: whether the blockage involves prevention of the appearance of the mitotic apparatus or its breakdown, whether its formation takes an abnormal course, whether it becomes disorganized once formed or merely is arrested at a given stage. These questions are dealt with by observations of the isolated mitotic apparatus obtained from blocked cells and by studies of time-concentration relations in blockage and recovery. The earlier observations on the eggs of *Dendraster excentricus* are confirmed in detail.

MATERIALS AND METHODS

Animals.—The shedding of eggs of *Strongylocentrotus purpuratus* was initiated by the intracoelomic injection of 2 ml of 0.5 *M* KCl. The animals were inverted over 150 ml beakers of sea water and placed at 5°C and allowed to shed for 20–30 mins. The eggs were then washed three times by decantation with 18°C sea water and used immediately. Sperm was obtained by allowing the males to shed into dry syracuse dishes at 5°C, and storing the "dry sperm" in the refrigerator.

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[1] This work was supported by grants from the Office of Naval Research and from the American Cancer Society.

[2] This work was carried out by the senior author during the tenure of a research professorship in the Institute for Basic Research in Science, University of California. The material support of the work came from the Office of Naval Research and from the American Cancer Society, whose assistance is gratefully acknowledged.

[3] Postdoctoral research fellow, U.S. Public Health Service.

Isolation of mitotic apparatus.—The technique described by Mazia [4] was used for the isolation of the mitotic apparatus. The following procedure was employed. Unfertilized eggs were placed into a sea water solution of 0.01 per cent crude protease (Worthington), previously equilibrated at 18°C, for nine mins. This procedure removed the vitelline membrane of the eggs. The protease solution was removed by gentle centrifugation and the eggs were placed into flat covered dishes, one egg layer thick, inseminated, and incubated at 18°C. At the desired time the eggs were treated with the mercaptoethanol. Finally, they were concentrated by gentle centrifugation, placed into cold 35 per cent ethanol at -10°C, and stored at this temperature. For isolation of the mitotic apparatus from the surrounding cytoplasm the eggs were placed into a 1 per cent solution of digitonin (Nutritional Biochemical Corporation). The external cytoplasm was almost immediately dissolved away leaving the clean mitotic apparatus (spindle-aster-chromosome complex).

Chemicals.—The 2-mercaptoethanol ($HSCH_2CH_2OH$) was obtained from Eastman Organic Chemicals, Rochester, N.Y. Fresh solutions of mercaptoethanol in sea water were made up daily, since it was determined that about 10 per cent of the mercaptoethanol was oxidized in sea water after 24 hours. At a concentration of 0.075 *M*, the mercaptoethanol had a pH of 8, very close to that of the normal sea water.

RESULTS

The Critical Concentration for Prevention of Division

Eggs were immersed in a series of concentrations of mercaptoethanol at 30, 60 and 75 mins after insemination. In *S. purpuratus* eggs metaphase is reached in 75 mins at 18°. At 60 mins, the cells have not reached full prophase and at 30 mins the pronuclei have joined and a monaster is present. The eggs were left in the mercaptoethanol solutions for 6–7 hours in order to distinguish between a definite blockage and a retardation of progress toward division. Our results on time-concentration dependence are assembled in Table I.

It is seen that full blockage occurs at a concentration of about 0.075 *M*, and that this concentration is fully effective whether applied at 30, 60 or 75 mins after fertilization. At this temperature normal cells go through karyokinesis at 85–90 mins after fertilization. As will be seen, the effect of 0.075 *M* mercaptoethanol is reversible.

Effects of Concentrations below the Critical Concentration

Dependence on time when mercaptoethanol is applied

If we consider, in Table I, the effects of 0.05 *M* mercaptoethanol when introduced at 30 and 60 mins, it is evident that its blocking effect is greater

Table I. *Mercaptoethanol concentration series.*

Time after insemination when eggs are treated ...	30 minutes		60 minutes		75 minutes	
	% cleaved 7 hours after insemination	Stage of development after 7 hrs. in ME	% cleaved 7 hours after insemination	Stage of development after 7 hrs. in ME	% cleaved 7 hours after insemination	Stage of development after 7 hrs. in ME
Concentration (molarity)						
0.5	0	cytolysis	0	0	0	0
0.25	0	cytolysis	0	0	0	0
0.1	0	cytolysis	0	0	9	2 cell
0.075	0	0	0.001	1 egg in 2 cell stage	10	2 cell
0.05	35	2 cell	81	2 cell	—	—
0.025	94	73 % 2 cell 31 % 4 cell	98	2 cell	—	—
0.01	—	—	98	4 and 8 cell stage now undergoing cytolysis	—	—
0.005	—	—	98	early blastula	—	—
0.001	95	4 and 8 cell stage, some 16 cell	—	—	—	—
Controls	100	early blastula	98	early blastula	98	blastula

when it is applied earlier. This observation, among others, argues against the hypothesis that the cells proceed normally to a given stage at which they become sensitive to the mercaptoethanol.

Later effects of subcritical concentrations

It is evident from Table I that there are later effects of lower concentrations of mercaptoethanol. At 0.05 M all of the cells that escaped blockage during the first division were blocked in the second. At 0.025 M, 70–80 per cent were blocked in the two-cell stage, and those that completed the second division were blocked before the third division. At 0.01 M, some of the cells went through the third division before being arrested. At 0.005 M, the embryos proceeded at least to the blastula stage at a normal rate. The question is whether the effect is cumulative in character or whether successive generations, involving smaller and smaller cells, become more sensitive to the mercaptoethanol.

This question may be answered by a simple test. Eggs placed into 0.05 *M* mercaptoethanol at 80–90 minutes after fertilization go through their first division and are blocked at the two-cell stage. Will 0.05 *M* mercaptoethanol block the second division if it is applied after the first has been completed? The experiment was carried out by placing *S. purpuratus* eggs into the 0.05 *M* solution at 115 minutes after first cleavage. It was observed that they completed the second division and were blocked before the third. Therefore, the second division is no more sensitive than the first, but rather this concentration is effective when applied over a period of more than one division.

Effects of the critical concentration

The stage at which mercaptoethanol is effective.—In the earlier study on *Dendraster* eggs [5] it was found that if the eggs had reached a rather well-defined point in their progress toward division before the mercaptoethanol was introduced it was without effect; the cells proceeded through division and were blocked before the next. The critical point was about the time of metaphase. The sharpness of the transition from full sensitivity to apparently complete insensitivity was stressed. These experiments were repeated in considerable detail in our present study on *S. purpuratus* eggs as a basis for the study, discussed below, of the morphological features of blockage with mercaptoethanol. Samples of eggs were placed in 0.075 *M* mercaptoethanol at five-minute intervals, beginning 60 mins after insemination. The temperature was 18.8°. The results are recorded in Table II. It is clear that in this

TABLE II. *Critical blockage.*

Time after insemination when immersed into 0.075 *M* mercaptoethanol	Progress at 85 minutes after insemination	Progress at 420 minutes after insemination
60	no division	0.01 % 2 cell
65	no division	0.05 % 2 cell
70	5 % 2 cell	10 % 2 cell
75	50 % 2 cell	55 % 2 cell
80	80 % 2 cell	85 % 2 cell
Controls Temp. 18.8°	80–90 % 2 cell	98 % morula

series of experiments (carried out at a slightly higher temperature than those given in Table I) the critical time of transition from full sensitivity to insensitivity was about 70 mins after fertilization. By the seventy-fifth minute, at least half of the cells had passed through the transition from full sensitivity to full insensitivity, and by the eightieth minute 85 per cent were insensitive. In other experimental series at 18° the critical point was at 75 mins after fertilization.

The data in Table II suggest that the division of those cells that had escaped the block proceeded in the mercaptoethanol without great delay. The number of cells in the control group that had divided at the eightieth minute was 80–90 per cent of the population. In the group that had been placed in mercaptoethanol at the seventy-fifth minute after fertilization, 50 per cent had divided by the eighty-fifth minute. It is seen in the third column that only 55 per cent of this population has passed into the insensitive stage. Therefore, those cells that were capable of dividing at all entered division no more slowly than the controls. The same argument applies to the group that was placed into mercaptoethanol at the eightieth minute. It would appear, then, that the transition from full sensitivity to insensitivity takes place very rapidly, and that the eggs that have made the transition are completely insensitive within limits of our ability to detect an effect. The accurate determination of division rates should, with appropriate statistical analysis, provide more definite information as to the actual time course of the apparently rather abrupt all-to-nothing transition.

Reversibility

The blockage of division by mercaptoethanol is completely reversible if the eggs are not exposed too long. The effects of exposures to 0.075 *M* mercaptoethanol for periods up to two hours have been investigated carefully in experiments where the reagent was introduced at 30 mins and at 75 mins after fertilization. The effects of exposures of up to 90 mins were reversible in all cases. Not only did over 90 per cent of the eggs cleave upon return to sea water, but early development proceeded normally. After two hours of exposure, a large proportion of the eggs cleaved, but about 50 per cent died before hatching. Thus, while this high concentration of mercaptoethanol can be considered to be relatively innocuous to the egg apart from its effect on mitosis, it is by no means entirely so. This conclusion is also supported by the results of experiments in which unfertilized eggs were immersed in 0.1 *M* mercaptoethanol before fertilization. After one hour of exposure, all of the eggs fertilized and divided at the normal time upon being returned to sea vater. After two hours, the time from fertilization to first cleavage was xtended from 90 to 120 mins. After three hours in mercaptoethanol, the ggs would fertilize and divide, but division was greatly delayed and visibly bnormal. In the course of these experiments, a number of interesting effects n the fertilization membrane was observed, which will be described in nother publication.

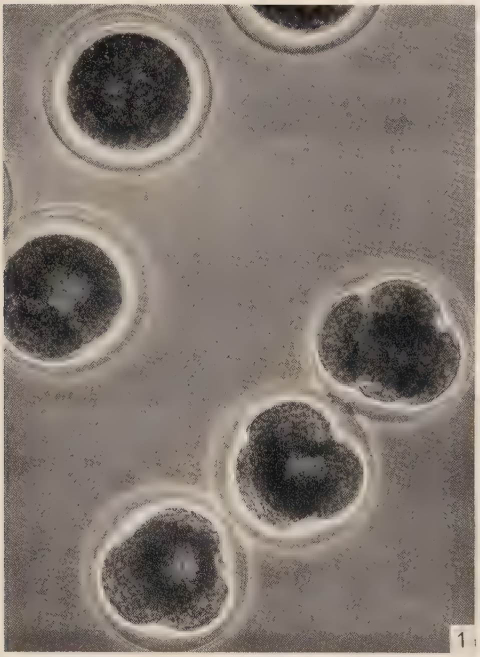
In the study on *Dendraster* [5], it was concluded that the mitotic block by nercaptoethanol had the character of an arrest; that is, the egg behaved as hough the egg resumed its progress toward division exactly at the point vhere it was when the reagent was applied. This question has been examined n some detail in the present study. If the mercaptoethanol "destroys" the leveloping mitotic apparatus, it would have to be reconstructed, and the total ime from fertilization to division, minus the time the egg spent in mercaptothanol, should be greater than the division time of the control cells. This vould be what Zeuthen (personal communication) has termed "excess lelay". If, on the other hand, the eggs proceeded in mercaptoethanol to a ritical stage at which the block set in, then the total time from fertilization o division, minus the time in mercaptoethanol, should be less than the ormal division time. If they were merely stopped at whatever stage they vere in, the division time should be the normal time plus the time spent in nercaptoethanol.

Experiments on this point were carried out as follows. Fertilized eggs were laced in 0.08 *M* mercaptoethanol at 55 mins after fertilization. Samples vere removed at 15, 30, 45 and 60 mins and the cleavage rates at 18° were ollowed carefully. Table III gives the results.

TABLE III. *Division times of eggs removed from 0.08 M mercaptoethanol after various periods of exposure.*

Units are minutes, temperature 18.0.

A Eggs placed in ME 55 minutes after fertilization and exposed for:	B Total time from fertilization to 50 per cent cleavage	Column B minus Column A	Excess delay
0 (controls)	90	90	
15	100	85	−5
32	124	92	+2
46	135	89	−1
60	155	95	+5



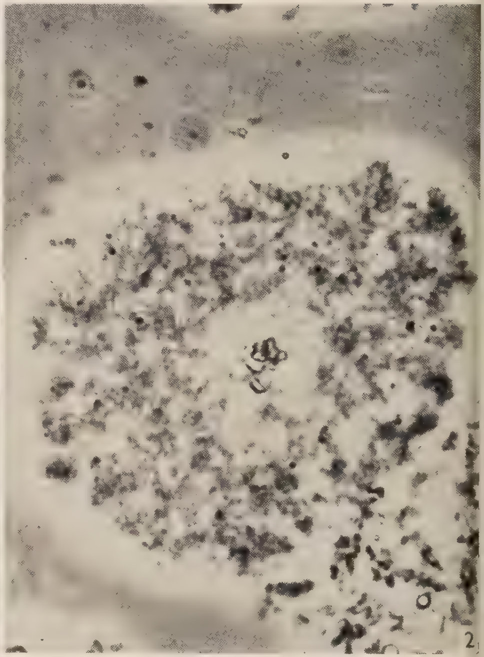


Fig. 1.—Cleavage of eggs that have been returned to sea water after exposure to 0.075 *M* mercaptoethanol beginning at 60 mins after insemination and terminating at 150 mins after insemination. Eggs are cleaving directly into four cells at 35 mins after return to sea water. (Phase contrast; low power.)

Fig. 2.—Mitotic figure isolated from cells that had been placed in 0.075 *M* mercaptoethanol at 60 mins after insemination.

This experiment at face value would tell us that the blockage by mercaptoethanol is simply an arrest. The apparent advances or retardations of a few minutes cannot be considered to be significant measurements; the estimates of cleavage rates are not that accurate. Either the mitotic machinery is merely "frozen" upon immersion in mercaptoethanol or else any changes that are produced are very rapidly reversed when the eggs are returned to sea water.

Nuclear reproduction during the mercaptoethanol block.—It was often observed in the earlier study on *Dendraster* that if the blocked eggs were left in the mercaptoethanol until the controls had completed the second division, they divided directly into four cells when returned to sea water. This behavior was confirmed and studied more carefully in *S. purpuratus*. If the eggs were placed in 0.075 *M* mercaptoethanol at 60 mins after fertilizaion, exposed for 60 mins or more and then returned to sea water, a large proportion of the eggs (at least 50 per cent and in some cases nearly 100 per cent) cleaved directly into four cells. Such cleavages are shown in Fig. 1. The embryos developed normally. A reasonable interpretation of this observation is that the reproduction of nuclear material and of centrioles was proceeding during the time the eggs were in mercaptoethanol. This would end to support the inference—inherent in the speculations which led to the use of mercaptoethanol—that the agent was acting mainly on the development of the spindle and asters. Swann (8) has described multiple divisions in *Psammechinus miliaris* eggs during recovery from blockage by ether, and interprets them in a similar way.

The Structure of the Mitotic Apparatus in Cells Blocked by Mercaptoethanol

The study of the isolated mitotic apparatus permits us to scrutinize morphoogical details rather easily, and to consider the question of the existence of he mitotic apparatus as a discrete body even when it has lost those optical attributes which ordinarily permit us to recognize it by fixation and staining, phase contrast microscopy, or polarization microscopy.

We have subjected populations of *S. purpuratus* eggs that had been placed in 0.075 *M* mercaptoethanol at 30, 60 and 75 mins after fertilization to the isolation procedure described in the section on methods. In all cases, a mitotic figure was isolable. The possibility that the mitotic apparatus dissolves under the action of mercaptoethanol is definitely excluded.

S. purpuratus eggs at 30 mins after fertilization are in a stage when the pronuclei have just joined. If they are left in mercaptoethanol for 60 mins and hen subjected to the isolation procedure, the nuclei are found to be in early prophase. Apparently at this stage the cells can proceed a short distance toward division while in mercaptoethanol. When the eggs are exposed at 60 mins after fertilization, a time when they have not yet entered prophase, hey proceed through prophase in the course of one hour but do not quite reach metaphase (Fig. 2). Thus nuclear events seem to have proceeded, even hough the aster structure is far less orderly than in an untreated egg.

If the eggs are treated at 75 minutes after insemination, when the cells are in metaphase (Fig. 3), the effect is that of a structural disordering. The appearance of the mitotic apparatus as isolated by exactly the same method as used for the controls in Fig. 3 is that of a mass whose fibers are no longer so distinct and regular, in which the chromosomes are no longer lined up sharply on a metaphase plate but rather lie somewhat randomly in the general area of the equator (Fig. 4). One no longer distinguishes between spindles and asters, but observes what can be described as a rather amorphous gel.

We may ask whether this action of mercaptoethanol on cells that are in methaphase and are just about to enter the mercaptoethanol-insensitive phase has the properties of a direct and immediate effect or whether it takes place gradually. To test this, eggs were placed in mercaptoethanol at 75 mins after insemination and removed and immersed in the cold ethanol at various times. As early as 10 mins after exposure, the isolated figures were disorganized as in Fig. 4, and there was little if any further change on longer immersion (Fig. 6). For comparison Fig. 5 shows the controls at 85 mins after insemination. We do not yet have information as to whether the structural consequences of the action of mercaptoethanol appear even more rapidly than in 10 mins. This part of our study had been designed primarily to determine exactly the structural status of the mitotic apparatus at the time when it was passing from mercaptoethanol sensitivity to insensitivity, and time was consumed in collecting the controls. Clearly the effect of mercaptoethanol when applied at the time of metaphase is describable as a disorganization of the structure of the mitotic apparatus at the microscopic level.

Preliminary results on effect of mercaptoethanol on oxygen consumption.— One of the questions raised by the effect of mercaptoethanol on mitosis is whether it operates directly upon the structure of the mitotic machinery or indirectly by way of energetic mechanisms. Relevant, if necessarily equivocal, information might be obtained by studying the effect of mercaptoethanol on the respiration of sea urchin eggs. A preliminary study was undertaken with the cooperation of Dr. Erik Zeuthen, of the Biological Institute, Carlberg Laboratory, Copenhagen, when he was a guest of our laboratory. *S. purpuratus* eggs were fertilized, washed three times to remove excess sperm, and loaded into Warburg respirometer vessels within 30 minutes after fertiliza-

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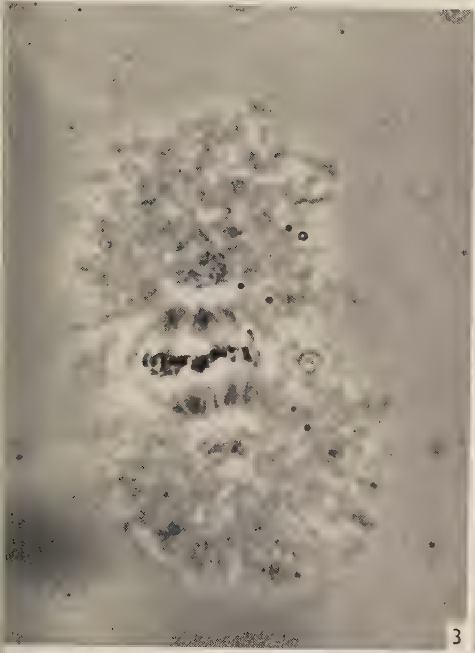
Fig. 3.—Isolated mitotic apparatus at 75 mins after insemination. 18°C. This is the stage of the mitotic apparatus at the time the samples shown in Figs. 4 and 6 were put into 0.075 *M* mercaptoethanol. (Phase contrast; 690 ×.)

Fig. 4.—Mitotic apparatus isolated after 10 mins exposure in 0.075 *M* mercaptoethanol beginning at 75 mins after insemination. This photograph is to be compared with Fig. 3, which shows the condition of the mitotic apparatus at the time mercaptoethanol was introduced. (Phase contrast; 690 ×.)

Fig. 5.—Normal mitotic apparatus at 85 mins after insemination. Comparing this with Figs. 3 and 4, we may compare the changes in the normal cell and in those exposed to mercaptoethanol from the seventy-fifth to the eigthy-fifth minute after insemination. (Phase contrast, 690 ×.)

Fig. 6.—Mitotic apparatus isolated after 27 immersion in 0.075 *M* mercaptoethanol, beginning at 77 mins after insemination. (Phase constrast, 690 ×.)



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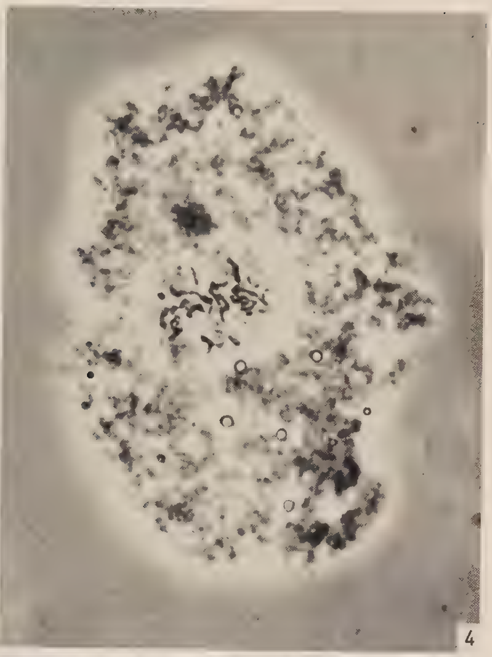

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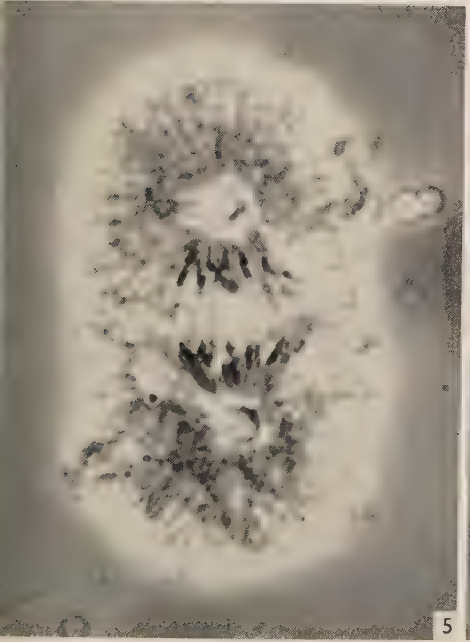

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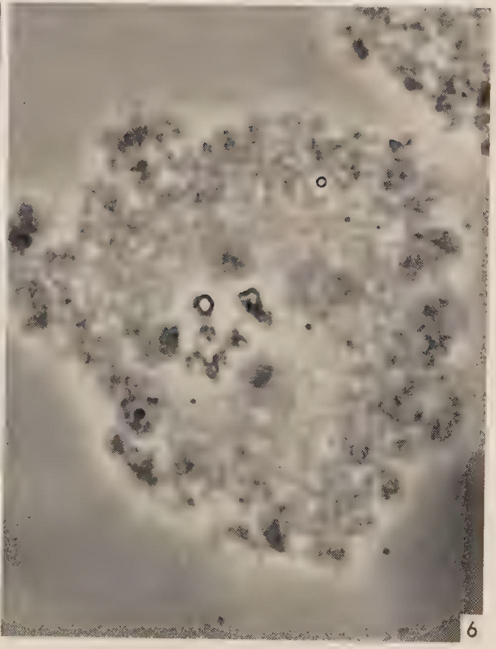

6

tion. The vessels contained NaOH in the center well and appropriate amounts of mercaptoethanol, dissolved in sea water, in the side arms. Controls in which the eggs were replaced with sea water were maintained in order to establish any correction for the slow but measurable auto-oxidation of mercaptoethanol. In order to establish a base line of normal respiration, the readings were continued for an hour after closing the respirometers by which time 1½ hours had elapsed since insemination. The mercaptoethanol was tipped into the vessels at about 1½ hours after fertilization, or the time of first cleavage. Thus, we were studying the effect of mercaptoethanol on respiration of eggs in the two-cell stage. The study was not extensive enough to warrant a detailed presentation of data, but the results were clear so far as the discussion in this paper is concerned. A concentration of mercaptoethanol, adequate to block division in 85 per cent of the population, 0.06 *M*, was found to lower oxygen consumption by about 40 per cent. A concentration of 0.03 *M*, which permitted the eggs to go through the second division but blocked them before the third division reduced the respiration by 10 per cent. A concentration of 0.01 *M*, which did not block division, showed no measurable effect on respiration. The observations of the effects on division were made on the eggs removed from the Warburg vessels at the end of the experiments, reassuring us that the measurements were not influenced by effects of crowding or mechanical damage due to shaking.

DISCUSSION

Mercaptoethanol was chosen for study because it had properties that would permit a biological test of predictions that follow from our present information concerning the intermolecular linkages by which the mitotic apparatus is assembled. There is now strong evidence that the SH groups of the constituent protein molecules play a predominant part in the assembly of this large structure, and it was predicted that introduction of a high concentration of SH groups in the form of a compound such as mercaptoethanol should interfere with the establishment of the protein-to-protein linkages and therefore block mitosis at the stage of the formation of the mitotic apparatus.

The results obtained with *Dendraster* eggs conformed to this prediction and are now confirmed and amplified in some details in the present studies using the eggs of another echinoid. The critical concentrations for blockage of the first division are not grossly different for the two forms, nor are the effects of lower concentrations in permitting a limited number of divisions to take place. What is perhaps the most interesting fact—that there is an abrupt ransition from full sensitivity to full insensitivity at a stage judged to be metaphase—has been confirmed, and the availability of the *S. purpuratus* eggs in large quantities for application of isolation techniques has permitted us to define this stage by accurate microscopic observation as well as by the clock.

The isolation experiments were carried out at a temperature and in lots of eggs in which the transition from sensitivity to insensitivity to 0.075 *M* mercaptoethanol set in a 75 mins after fertilization. Fig. 3 shows the stage of the mitotic apparatus at this time. It is clearly metaphase. We are not certain how long these cells pause in metaphase and therefore cannot say whether the critical point is the beginning or end of this short period, but it most be short because these cells are in full anaphase, with chromosomes well on their way to the poles, 10 mins or later, as is shown in Fig. 5. We can imagine that within a few minutes after the stage shown in Fig. 3 the structure is "locked" in some way so that it is no longer sensitive to whatever the mercaptoethanol is doing.

In the previous paper, there was a rather lengthy discussion of whether the blockage constituted an arrest at the critical stage and of whether the mercaptoethanol acted on the structure of the mitotic apparatus or through some other effect on the cell. Concerning the former question, the evidence n favor of an arrest—in contrast to regression or breakdown of the mitotic apparatus—was the observation that the exposure to mercaptoethanol produced no net delay. The cell behaved as if nothing had happened while it was immersed in mercaptoethanol; the total time from fertilization to division, minus the time spent in mercaptoethanol, was equal to the normal division time. This fact was confirmed for the eggs of *S. purpuratus* when exposed at metaphase to 0.08 *M* mercaptoethanol for periods up to an hour (Table III).

The idea of a simple arrest led us to expect that we would isolate normal metaphase figures from such eggs. In Figs. 4 and 6 we see that this is clearly not the case. Had the morphological observations been made first, we would have predicted a measureable excess delay, for the metaphase figures appear to have become badly disorganized after 10 minutes in mercaptoethanol and perhaps sooner. Every feature characterizing metaphase (with he possible but not certain exception of the condensed state of the chromosomes) appears to have been destroyed. The poles are poorly defined, the asters are difficult to distinguish from the spindle, and the mitotic figure as a whole has lost its sharply fibrous character. While suggestions of the form of the original mitotic apparatus persist, the dispassionate observer would probably describe Figs. 4 and 6 as showing amorphous masses of material in which chromosomes are found in disorderly arrangement. We recognize the masses as being the mitotic apparatus because their size corresponds, because they do contain chromosomes, and because we know from the experience of this laboratory that everything but the mitotic apparatus is dispersed by our isolation procedure.

The observations on eggs placed in mercaptoethanol at earlier times show that the approach of the chromosomes to the prophase conditions continues, but again the structure of the monasters is much less orderly than would be the case in control cells at this time. That the nuclear cycle is relatively insensitive to mercaptoethanol is even more strikingly suggested by the finding that cells whose division has been blocked for a sufficiently long time divide directly into four apparently normal daughter cells but we have done no detailed work to determine whether we are dealing with some kind of endomitotic mechanism.

The evidence for a simple arrest (which turns out to be a misleading term) at metaphase by mercaptoethanol applied at that stage (Table I) might seem to be in contradiction with the observation that mercaptoethanol causes a serious disorganization of the metaphase figure. To bring them into concord, we must assume that the disorganization is reversed in a very short time, a matter of minutes, when the egg is returned to sea water, and we plan to follow the stages of this recovery process in detail. One hypothesis that can be made is that the disorganization that is so striking to the eye would not be nearly so drastic if we could view it more closely. Let us imagine, for instance, that the metaphase configuration of the chromosomes is the equilibrium condition brought about by tension of the chromosomal fibers, the unseparated chromosome pairs being subjected to pull from the two poles. Such an interpretation of the meaning of the metaphase picture has been proposed by Oestergren [6]. Let us suppose that the effect of mercaptoethanol is to loosen intermolecular bonds responsible for the condensed and oriented arrays that we see as microscopic fibers. If the fibers seen in fixed preparations, by phase contrast optics, or by polarization optics are indeed accounted for as "condensed regions of higher orientation" in what is essentially a gel, as has been proposed [4], the intermolecular dissociation might not have to go very far before the originally oriented and condensed regions would no longer be identifiable against the background of the similar material in a less condensed condition. The molecules might remain sufficiently close together or might be held together by a smaller but adequate number of bonds so that they were in a position to reassociate readily in their condensed and oriented condition when the mercaptoethanol was removed. The "loosening" of bundles of molecular chains might well be imagined to have the effect of a "relaxation" so far as the chromosomal fibers are concerned, permitting the chromosomes to be displaced from their rigid metaphase configuration. The tightening upon reversal might have the effect of a contraction pulling them back into this configuration. Alternative speculations are possible of course. What has to be accounted for is the apparent preservation of the underlying organization of the mitotic figure where it appears at the microscopic level to be highly disorganized. These observations recall the contrasting effects of abnormal temperatures on the meiotic spindles of *Chaetopterus* eggs, as described by Inoué. Inoué [2] observed that colchicine appeared to disperse this spindle completely, with scattering of the chromosomes, and that it required three and a half hours to reform. On the other hand, abnormally high and low temperatures [2] had a similar observed effect, but recovery at normal temperatures was completed within 15 minutes.

The newer experiments throw no light on the baffling problem of what happens to the mitotic apparatus shortly after metaphase to account for the insensitivity to mercaptoethanol, except to permit us to state the problem in erms of its instantaneous structure as well as of its ability to proceed through he mitotic process. Somehow, the structure is "locked" at the critical time.

In the earlier paper, the question of a direct action of mercaptoethanol of the structural associations responsible for the organization of the mitotic apparatus was discussed in contrast with an indirect action by way of energetic requirements of various stages. This question is posed by the fact that the periods of sensitivity and insensitivity to mercaptoethanol correspond roughly, though not in detail, to the periods of sensitivity and insenstitivity to a variety of metabolic inhibitors. Our preliminary experiments on the effects of mercaptoethanol on cell respiration do not resolve this problem. We cannot lightly discount our observation that respiration was 30 per cent of normal at a concentration of mercaptoethanol that blocked cleavage and 90 per cent of normal at a concentration that permitted one cleavage but blocked the subsequent one. Consulting the data on the relations between cell respiration and cleavage of sea urchin eggs compiled by Krahl [3], it is worthy of remark that most of the inhibitors that he lists do not completely block division unless the inhbition of respiration is greater than 50 per cent. Cyanide does not inhibit division completely until respiration is depressed to 30 per cent of normal and the same figure is given for inhibition of cleavage by CO, the agent used in an excellent study by Swann [7] that was discussed in the previous paper of this series. Thus it is quite conceivable that the CO effect, which requires 70 per cent inhibition of respiration, should not be interpreted in the same way as that of mercaptoethanol, and that the latter does not operate indirectly through the energy supply. We are compelled to recognize, however, that oxygen consumption is only a very superficial indicator of events involving more basic energy transfers, and must still leave open the possibility that the effect of mercaptoethanol is an indirect one.

The question whether the effect is a direct one on the mitotic apparatus or an indirect one is of moment in relation to the hypothesis that motivated these studies in the first place, but is now separable from the question whether the blockage is ultimately an effect on the gross structure of the mitotic apparatus. Clearly this is so. The observations are entirely consistent with the prediction that mercaptoethanol would act directly by splitting or interfering with bonds in the mitotic apparatus involving thiol groups. Since we now know that the blockage involves a manifest disorganization of the structure of the mitotic apparatus, the alternative hypothesis that it acts indirectly through the energetic mechanisms would now imply that the structure of the mitotic apparatus at metaphase was a dynamic one depending on a continuous energy supply. This is by no means an unattractive possibility. In fact, the general question of the relation between the stability of intracellular structures and the cell's energetic mechanisms demands somewhat more effective consideration than it has received.

SUMMARY

1. The cleavage of fertilized eggs of *Strongylocentrotus purpuratus* may be blocked reversibly by mercaptoethanol (monothioethylene glycol).

2. The critical concentration for blockage of the next division is about 0.075 *M*. At lower concentrations, the eggs may be blocked after one or more divisions.

3. The divisions are blocked, at the critical concentration, if mercaptoethanol is introduced at any time up to metaphase. If it is introduced shortly after metaphase, cleavage proceeds without delay and the cells are blocked in the following division.

4. Superficially, the blockage has the character of a simple arrest. That is, the total time from fertilization to division, minus the time spent in mercaptoethanol, is equal to the division time of the controls. However, the following observations indicate that the evident arrest is not merely a "freezing" of the mitotic apparatus at a given stage.

5. Nuclear reproduction and possibly the reproduction of the centrioles roceed during the mercaptoethanol block. If the eggs are removed from nercaptoethanol after the time when the controls have made their second livision, they divide directly into four cells, from which a normal embryo levelops.

6. Isolation of the mitotic apparatus from the blocked cells shows that heir structure at the microscopic level is very much disorganized by mer-aptoethanol.

7. It is concluded that the apparent disorganization at the microscopic evel does not imply an equally drastic disruption of the underlying organi-ation. The effect of mercaptoethanol is analogized to a "relaxation" or 'loosening" of structure that can be reversed almost instantaneously.

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INVESTIGATIONS ON THE MECHANISM OF ADAPTATION TO SHOCK INDUCED BY DISTILLED WATER

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Received August 28, 1957

Ehrlich's mast cells contain histamine as well as heparin [21, 22, 7, 3]. This is particularly shown by the high values found in mast cell tumours (urticaria pigmentosa). The same conclusion may be drawn from the experiments of Cass *et al.* who found very high histamine values in the mast cell tumours of dogs.

Numerous authors [10, 11, 4, 19, 18] have observed that the administration of either histamine liberators or of distilled water was followed by symptoms of histamine shock in rats. After intraperitoneal injection of these substances, the histamine content of the peritoneal fluid increased. At the same time the mast cells became swollen and degranulated. Further administration of histamine liberators resulted in milder symptoms, and only a minor degree of shock was elicited even a month after the first injection. Keller and Burkard [18] have suggested that the reduction in the degree of shock is due to degranulation and partial destruction of the mast cells, as a result of which histamine can no longer be released from them.

This explanation does not seem to us to be entirely satisfactory because of reports on experiments with contrary results. The investigations of Halpern and Briot [12, 13] show that the replenishment of tissue (skin) histamine after depletion by dextran, a histamine liberator, takes only five days. Also, as the authors have shown, the restoration of the "chemically extractable" tissue histamine does not mean that it is at the same time "physiologically available" histamine. Bhattacharya and Lewis have also furnished evidence against Keller and Burkard's theory by showing that 12 days after the shock induced by distilled water the mast cells were completely normal as far as their numbers and qualities were concerned; in spite of this perfusion of a limb with a histamine liberator at this time caused no histamine release.

Consequently we have assumed that the failure of the appearance of histamine shock is not to be explained solely by the depletion of tissue and mast cell histamine, but might be due to the increased production of "resistin" described by Karady *et al.* [14, 15]. Resistin is a substance with antihistamine activity and its increased production, which occurs mainly after various stress conditions, renders the organism resistant to the effects of histamine.

In order to clarify this problem, experiments were carried out to answer the following questions:

(1) Is the intraperitoneal injection of distilled water followed by the symptoms of histamine shock due to histamine release from the peritoneum? What alterations occur in the mast cells of the peritoneum and the subcutis?

(2) Is the tissue histamine content depleted after intraperitoneal administration of distilled water? Does the depletion of histamine account for the development of resistance to the histamine releasing effects of further doses of distilled water?

(3) Is there any connection between the resistin content of the blood of animals shocked by distilled water, and the resistance of these animals to subsequent doses of histamine releaser?

METHODS

Twenty rats weighing 140–200 g, male and female, were used. Ten of them served as controls. Ten rats were given 20 ml of distilled water intraperitoneally and sacrificed, by destroying the medulla oblongata, at different intervals, namely 30 minutes and 1, 5, 11, 12, 13, 14 days respectively, after the intraperitoneal injection. The controls were injected with 20 ml of Tyrode solution intraperitoneally and were sacrified in the same manner and at the same intervals. From the animals sacrificed 30 minutes after the intraperitoneal injection of distilled water and Tyrode solution, respectively, 2 ml of peritoneal fluid were withdrawn before their killing, in order to determine the histamine content of the fluid. In addition, histological examinations of the peritoneum and subcutis of all experimental animals were carried out. The tissue pieces were mounted on a slide, allowed to dry and, without having been fixed, stained with a 1 per cent solution of toluidine blue in methyl acohol, for 1.5 minutes.

The content of tissue histamine of the subdermal tissue and peritoneum in all animals was extracted by the method of Barsoum–Gaddum [2] and Code [8], in Bikich–Borbola–Hetenyi's modification [5] and determined by the usual technique on atropinized guinea-pig's ileum. The peritoneal fluid of the animals sacrificed 30 minutes after the intraperitoneal injection of distilled water was essayed for histamine in the same manner.

In another group of experiments the serum of forty-two rats weighing 170–240 g, male and female, was investigated for its resistin content by a biological method described by Prokai, Mustardy and Karady [20]. The animals were sacrificed by exsanguination, at different periods, that is 1, 2, 3, 4, 5, 7, 11, 13 and 15 days after the intraperitoneal injection of 20 ml of distilled water. The sera of these rats were then transferred to guinea pigs which had previously been tested by means of histamine aerosol technique for histamine sensitivity, in order to see whether, due to the transferred serum, the histamine spray time of these guinea pigs had changed in relation to the resistin content of the transferred serum [20].

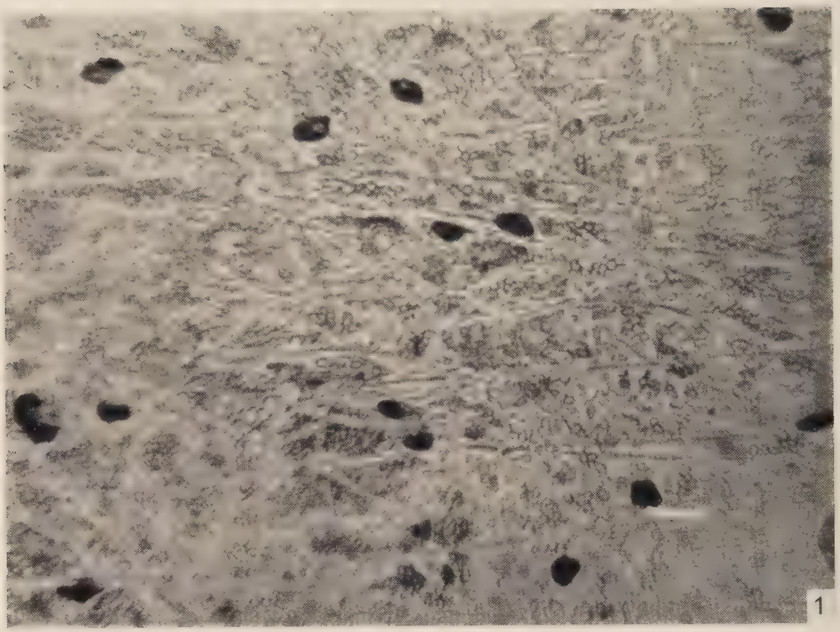

Fig. 1.—Mast cells of peritoneum in untreated animal. × 56.

RESULTS

The intraperitoneal injection of 20 ml of distilled water into rats produced erythema of the paws and nose after 5–10 minutes, which was followed by a generalised itching of the skin. In 20–30 minutes conspicuous oedema of the nose, ears, paws and genital organs had appeared. Subsequently the animals became dyspnoeic, prostrated and cyanosed, and the temperature dropped 1–3°C. The symptoms of shock then slowly regressed and disappeared after 6–8 hours.

The histamine content of the ascitic fluid was 2, 2.5, 2.4, 1.6 μg/ml. These data agree with those of Keller and Burkard. Histological examination of the peritoneum of untreated rats revealed that the mast cells were evenly distributed. In some places, however, they formed large groups adjacent to the blood vessels. Their oval and round cytoplasm was filled with granules which stained metachromatically. In the peritoneum of the rats killed 30 minutes after the injection of distilled water, the mast cells displayed enlargement, degranulation and disruption, while the subcutaneous tissue mast cells showed no significant changes at this time. In the peritoneum of the rats treated with Tyrode solution the mast cells were generally slightly swollen and some of

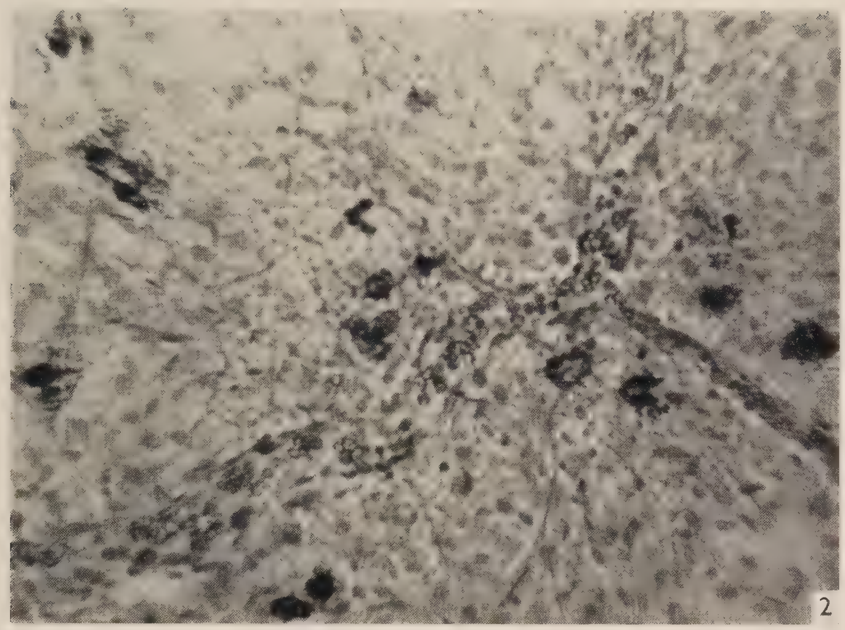
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Fig. 2.—Mast cells of peritoneum in animal treated with distilled water. × 56.

hem were disrupted. Similar changes were observed in the mast cells of the subcutaneous tissue 24 hours after treatment. Two weeks after production of the shock the histological appearance of the tissues was almost normal (see microphotographs, Figs. 1–3).

The results of the tissue histamine determinations are summarized in Table . The histamine content of the peritoneum declined during the first 24 hours but five days after the shock it had resumed its normal value. The histamine content of the subcutaneous tissue was slightly above normal.

It has been shown that the resistin content of the serum is accurately reflected by its protective action on guinea pigs subjected to the experimental inhalation of histamine sprays. The resistin content of the sera of rats killed at different periods after they had been shocked by the administration of distilled water is shown in Table II and Fig. 2. The sera of rats killed between the 3rd and 5th days after the shock, had very little effect on the spray time. From the 6th until the 15th days a pronounced prolongation of the spray time occurred. We have not yet obtained any data on the effects produced on the spray time by sera from rats treated more than 2 weeks previously with distilled water.

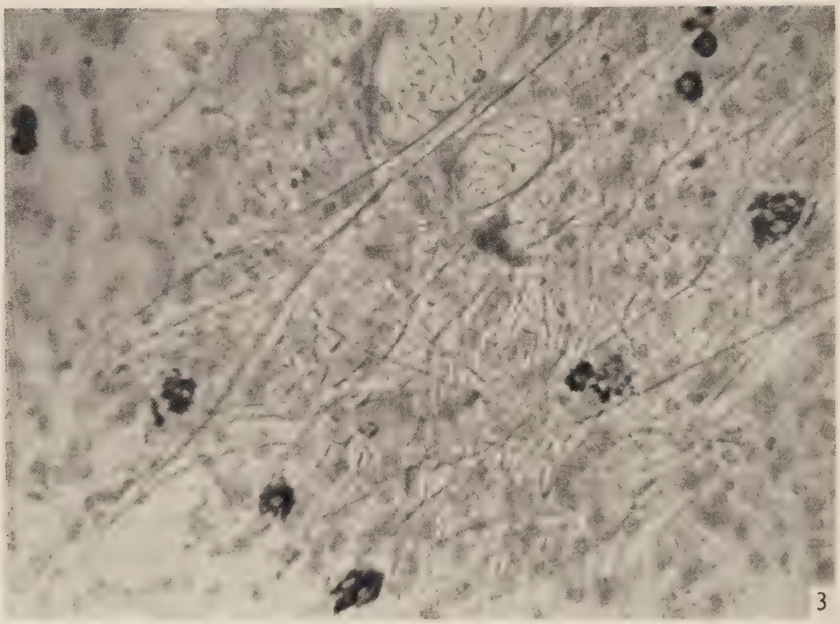

Fig. 3.—Mast cells of peritoneum in animal treated with Tyrode solution. × 56.

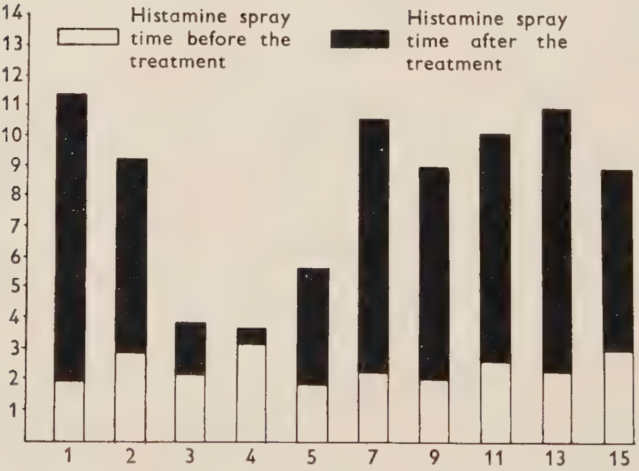

Fig. 4.—Prolongation of the histamine spray time after the shock due to distilled water. *Ordinate:* Time of histamine spray in minutes. *Abscissa:* Number of days elapsed since the shock produced by distilled water.

TABLE I. *Values of the determination of tissue histamine.*

Treatment	Time of killing after treatment	μg histamine per gram of tissue	
		Peritoneum	Subcutis
Distilled water	30 min.	7.4	28.7
	30 min.	6.9	26.8
	1 day	2.4	33.0
	1 day	2.6	31.0
	5 days	7.3	31.4
	5 days	6.7	30.5
	11 days	7.5	27.6
	12 days	7.3	31.5
	13 days	7.7	28.2
	14 days	7.1	28.5
Tyrode solution	30 min.	7.1	28.6
	30 min.	7.5	29.9
	1 day	4.8	27.8
	1 day	5.6	28.2
	5 days	7.3	31.3
	5 days	7.0	32.7
	11 days	7.5	25.8
	12 days	7.2	29.5
	13 days	6.8	30.8
	14 days	7.3	31.2

DISCUSSION

It has been shown that the intraperitoneal administration of distilled water esults in destruction of mast cells, histamine release, and the production f histamine shock. In the present experiments we have shown that the shock, roduced by distilled water, which arises as the result of the release of histanine, cannot be repeated for some time after the initial occurrence of the hock.

Our results are in complete agreement with the experimental data of Felderg and Talesnik [11], Fawcett [9, 10], Bhattacharya and Lewis [17], Merenthaler and Paff [19], Keller and Burkard [18]. We have observed similar ut less pronounced changes in the mast cells, when Tyrode solution was njected intraperitoneally instead of distilled water. Analogous data are pubished in a recent paper by Asboe-Hansen and Wegelius [1].

Table II. *Histamine spray time of guinea-pigs before and after the intraperitoneal injection of the serum of rats treated with distilled water.*

Number of days elapsed from the injection of distilled water to the death of the rats	Histamine spray time		
	Before treating	After treating	Group
1	1 min. 55 sec.	14 min. 30 sec.	1
1	2 15	8 25	
2	2 55	9 15	
3	2 00	4 20	2
3	2 20	3 30	
4	2 25	4 20	
4	4 00	3 00	
5	2 10	2 20	
5	1 50	11 40	
5	1 40	3 00	
7	1 30	12 05	3
7	3 15	9 15	
9	2 10	9 10	
9	2 00	8 40	
11	3 10	11 10	
13	2 30	12 45	
15	3 55	8 55	

P for groups 1 and 3 together < 0.01; P for group 2 > 0.1.

The reason why repeated aministration of histamine liberators does not lead to histamine release is not yet clear. It has been suggested that the depletion of histamine in the mast cells and other tissue cells is responsible for the absence of further release of histamine [18].

This explanation is plausible in the case of administration of Compound 48/80. Feldberg and Talesnik observed that the administration of Compound 48/80 caused a decrease in the histamine content of skin and muscles to about 10 per cent of its original value, and that it was not restored to its normal value until 45 days after administration of the drug. Similar results have been obtained by Briot (personal communication), who found that the histamine content of rat's skin was still less than its normal value 3 weeks after the commencement of administration of 48/80.

In the case of histamine liberators, distilled water and dextran, the situa- on is entirely different with reference to the replenishment of tissue histamine. eller and Burkard did not determine tissue histamine values in their experi- ents. We have shown, however, that the histamine content of the peritoneum nd other tissues, decreased as a result of shock induced by distilled water, eached its normal value again on the 5th day. Halpern and Briot state that, the case of dextran, replenishment of tissue histamine is complete in 4–5 ays. Under these conditions, in spite of normal histamine levels, the admi- istration of repeated doses of histamine liberators does not produce release f histamine. We have found this to be the case with distilled water, and Hal- ern and Briot have found it to be so with dextran.

These results imply that the cause of refractoriness of the organism treated ith a histamine liberator against further exposure to a histamine liberator annot be due entirely to the histamine depletion of the tissues. The absence f a satisfactory explanation of this phenomenon emphasizes the importance f our experiments. The resistin production of an animal refractory to the ction of histamine liberators is significantly increased. It is possible that the ncreased resistin production caused by the stress of histamine release, not nly renders the organism resistant to the effect of histamine (as we have shown reviously), but is also responsible for the inhibition of new histamine forma- ion in the tissues. Thus the resistin may prevent in some way the transforma- ion of "chemically extractable" histamine into a "physiologically available" orm. This suggestion, however, requires further investigation.

SUMMARY

1. After intraperitoneal injection of distilled water into the rat, symptoms f histamine shock occur, and the histamine content of the peritoneal fluid ncreases. At the same time, the mast cells of the peritoneum become swollen, egranulated, and disrupted. Similar changes occur in the subcutaneous issue after 24 hours. Injection of Tyrode solution has a similar though milder ffect.

2. The histamine content of the tissues does not change significantly.

3. A pronounced increase in the resistin content of the blood was demon- trated in animals treated with distilled water.

4. The increased production of resistin in animals subjected to stress by istilled water or other histamine liberators may be responsible for their efractory state towards histamine liberators.

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THE TIME SEQUENCE OF EARLY EVENTS IN THE FERTILIZATION OF SEA URCHIN EGGS

I. THE LATENT PERIOD AND THE CORTICAL REACTION[1]

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Received November 8, 1957

The first obvious indication of fertilization in eggs of the sea urchin is the elevation of a fertilization membrane about 30–35 seconds after insemination (cf. [12]). This event is preceded by breakdown of the cortical granules [11, 15] and an alteration in surface color when the eggs are viewed with dark-field illumination [16, 19]. If sperm attachment occurs in some eggs within a few seconds after insemination, and membrane elevation is delayed until 32 or 33 seconds, it must mean that a substantial latent period exists either between sperm attachment and cortical granule breakdown, or between granule breakdown and membrane elevation. A decision between these alternatives would be of considerable importance, since a substantial latent period between sperm attachment and granule breakdown would indicate the necessity of a block against polyspermy more rapid than the visible phase of the cortical reaction, as proposed by Rothschild and Swann [18].

It would probably not be possible to decide by observation of single eggs how closely in time these visible surface changes, to be referred to here as the "cortical reaction", follow upon successful sperm attachment, for the fertilizing spermatozoon cannot immediately be distinguished from many others which appear to be as firmly attached to the egg surface; moreover, at the high magnifications required to observe cortical granules, only a small segment of the egg surface can be examined at a time. For this reason, the chances of observing the entire sequence of events in the fertilization of a single egg are exceedingly small.

Recently, methods have been devised for determining statistically the time at which the fertilizing spermatozoa attach to the surfaces of a population of eggs [18, 9]. The best method for doing this at present is interruption of fertilization by the detergent sodium lauryl sulfate, as described by Hagström and Hagström. To this method has been added a second interruption technique by which eggs are fixed in formalin before, during, and after the cortical reaction in order to determine at different times after insemination what percentage of a population of eggs has begun, or has completed the cortical reaction. Thus with a pair of very simple statistical methods, it has been possible to determine accurately the duration of the latent period between sperm attachment and cortical granule breakdown and of the cortical reaction itself.

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[1] This investigation was supported by a Research Grant (C-3022(C)) from the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Maryland.

MATERIAL AND METHODS

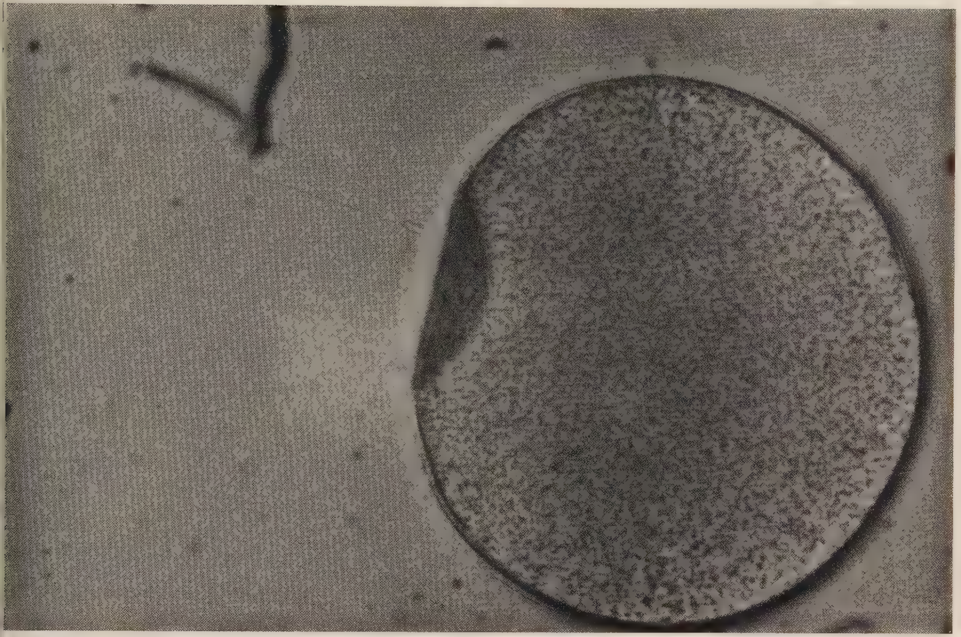
Eggs of the sea urchin, *Psammechinus miliaris*, from the Swedish west coast were most useful in this investigation, because the transparency of their cytoplasm made it possible to observe the cortical granules on nearly the entire surface of the egg, even after fixation by formalin. *Arbacia punctulata* eggs, collected in the region of Woods Hole, Mass., were not very favorable material for this study: however, it was possible to confirm in a general way the results obtained on the Swedish sea urchin material.

Eggs with jelly intact were inseminated with 10^7 sperm/ml to ensure rapid fertilization: at intervals of 3 seconds or more, aliquots of fertilizing gametes were transferred into 0.001 per cent sodium lauryl sulfate (at e.g. 4, 8, 12 and 18 seconds) and into 4 per cent formalin (at e.g. 16, 20, 24, 28, ... 60 seconds). After one minute, the eggs treated with sodium lauryl sulfate were washed with sea water. Formalin-fixed eggs tended to adhere strongly to glass and undergo changes in adhesiveness during and after the latent period. Trying to dislodge adhering eggs resulted in a systematic sampling error and some injury to the eggs: therefore, squares of glass (cut from microscope slides) were placed in the formalin dishes, and counts were made of eggs adhering to the glass squares. Counts of the percentage of eggs having begun a cortical reaction were made on about 150 eggs under a 50 × water immersion objective, with which the entire surface of each egg was scanned. The task was somewhat simplified by the fact that the partial elevation of a fertilization membrane in formalin resulted in a flattening of the underlying egg surface which was very easy to observe (see Fig. 1). Criteria for completion of the cortical reaction were: (1) completion of membrane elevation over the entire surface, or (2) disappearance of some cortical granules from all parts of the egg surface. (Often a few granules remained after the membrane had become elevated.) Many eggs which were fixed just after completion of the cortical reaction lost their membranes on the slightest agitation.

RESULTS

The Influence of Formalin on Psammechinus *and* Arbacia *Eggs*

Four per cent formalin penetrates *Psammechinus* and *Arbacia* eggs rapidly enough to preserve their structure and optical properties very well. This is



ig. 1.—An egg of *Psammechinus miliaris* interrupted by formalin fixation during the conducion of the cortical reaction 24 seconds after insemination at 20°C. Note the concavity on the egg urface under the fertilization membrane.

specially true of *Psammechinus* eggs, in which even the cortex is so well reserved as to give the observer the impression that they are alive. One triking alteration on fixation is the increase in adhesiveness previously nentioned. In *Arbacia* eggs, elevation of the fertilization membrane was nhanced by dilution of the formalin in sea water with about 20 per cent istilled water for fixation.

In living eggs there is a lag between the breakdown of cortical granules n any part of the egg surface and the elevation of the fertilization membrane n that area. Formalin evidently stops the cortical granule breakdown intantaneously, but does not interfere with subsequent elevation of the memrane on the surface where the cortical granules have broken down. For this eason eggs which were fixed during the cortical reaction show highly elevated nembrane blisters which extend nearly to the limit of the surface covered y the cortical reaction ("fertilized surface"). The flattening of the egg surface nder such partial membranes is presumably caused by the colloid osmotic ressure which accompanies membrane elevation (see Fig. 1).

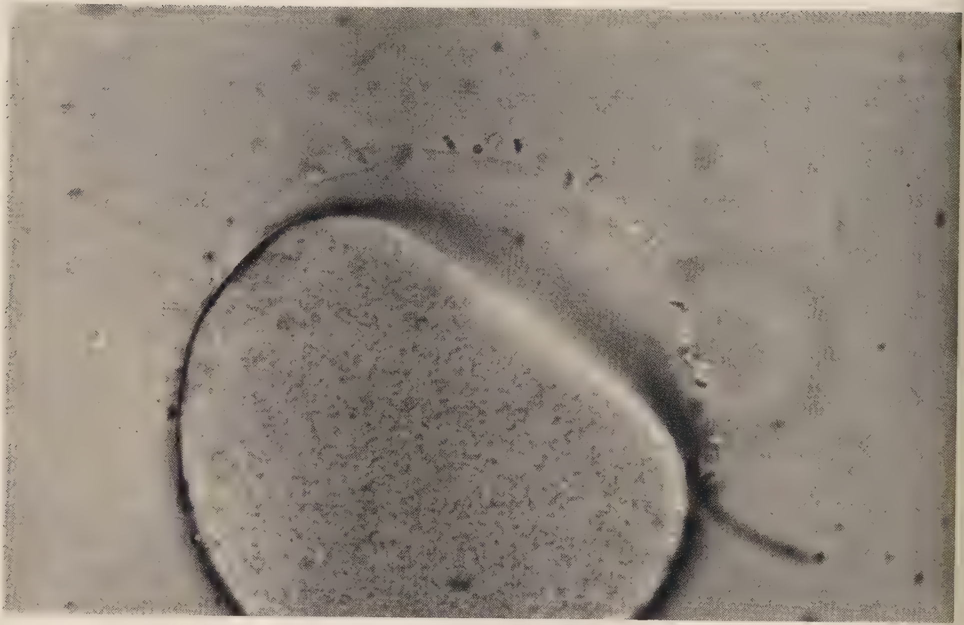


Fig. 2.—A *Psammechinus* egg fixed 32 seconds after insemination. Note the shape of the egg and the fact that the sperm is embedded in the egg cortex but has not entered.

Psammechinus eggs viewed under dark-field illumination showed the typical yellow color characteristic of the unfertilized surface even after formalin fixation. Parts of the surface that had been covered by the cortical reaction were pale white, as in the living egg.

The Duration of the Latent Period

The results of 15 experiments on *Psammechinus* and 6 on *Arbacia* eggs to determine the duration of the latent period are presented in Table I. The average duration of the latent period in *Psammechinus* was 17.6 seconds at 20°, with a spread of from about 12 to 22.6 seconds. In *Arbacia*, values of 17–22 seconds were obtained at 22°C. The opacity of the cytoplasm in the latter species rendered a large proportion of the cortical granules invisible; for this reason the values obtained may be a few seconds too high. From Table I it can be seen that the accuracy of the early experiments was poor. The situation improved when the glass squares were introduced into the formalin beakers to reduce the small but systematic sampling errors caused by differences in egg adhesiveness during the early seconds after fertilization.

TABLE I.

Experiment No.	Species	Latent period	Cortical reaction	Temperature (centigrade)
1	*Psamm.*	32–37	—	10
2	,,	18–22	—	15
3	,,	14–16	—	20
4	,,	11–13	—	20
5	,,	40	20–37	10
6	,,	22.6	24.5–26	20 (control)
7	,,	21.0	—	20 (periodate)
8	,,	19.0	20	20
9	,,	14–16	—	20
10	,,	40	29	10
11	,,	17.2	20.4	20
12	,,	16.8	—	20
13	,,	19.4	—	20
14	,,	18.5	—	20
15	,,	20.5	—	20
16	*Arbacia*	~20	—	22
17	,,	~18	—	22
18	,,	~22	—	22
19	,,	17–23	—	22
20	,,	~23	—	22
21	,,	62–67	—	11

In the later experiments the precision of the method was a few tenths of a second in determination of the latent period. There certainly was variability n the duration of the latent periods in different lots of eggs from different females, and apparently variability within several lots. Fig. 3 shows some data from which the latent period could be determined.

The Duration of the Cortical Reaction

In the duration of the cortical reaction as well as that of the latent period there was wide variation among lots of eggs (see Table I). Because of the amount of labor involved in searching several hundred eggs for remaining cortical granules, and because this task was easier in some relatively transparent batches of eggs, only a few experiments were counted for cortical reactions completed. At 20°C, two of the batches of *Psammechinus* eggs showed average cortical reaction durations of 20.0 and 20.4 seconds, very close to the values obtained by Rothschild and Swann with a second or two variation. A third experiment produced a longer duration, with a variation of about 1.5 seconds between rapidly and slowly fertilizing eggs: 24.5–26 seconds. An even greater variation in durations was observed in one of the experiments carried out at 10°C, where a spread of 20–37 seconds was found. This variation was clearly not due to fluctuations in temperature, as the eggs were stirred constantly during insemination.

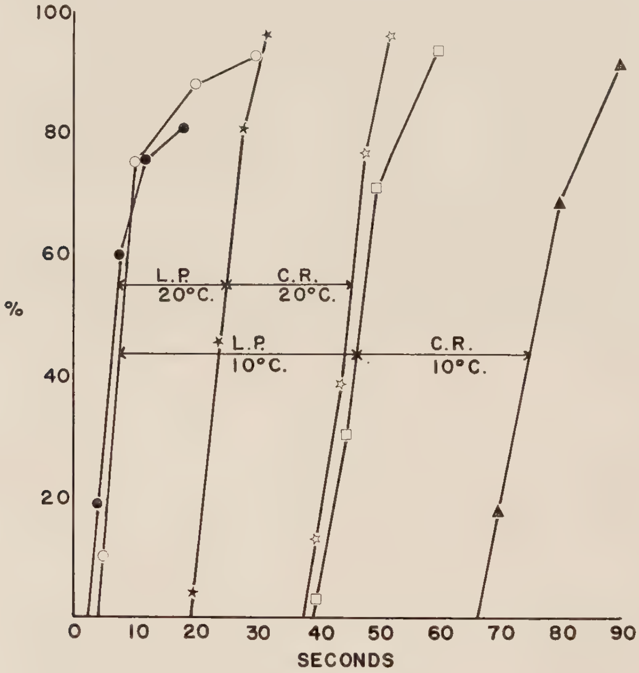


Fig. 3.—A representative experiment in which the duration of the latent period and cortical reaction were measured (see Table I, experiments 10 and 11). (○) and (●) are fertilization rate curves showing the time of sperm attachment at 10° and 20° respectively; (□) and (☆) show the percentage of eggs showing cortical reactions in progress at 10° and 20°; and (▼) and (✶) show the percentage of eggs which have completed cortical reactions at 10° and 20°.

The Time of Sperm Penetration

Formalin-fixed eggs exhibiting membranes over less than one fourth of their surface frequently had the fertilizing spermatozoan pulled away from the egg cortex by the elevating membrane after fixation. Eggs fixed in later stages of cortical conduction showed the anterior quarter or third of the sperm head embedded in the cortex, the tail in the perivitelline space held fast by he fertlization membrane (Fig. 2). It was evident that the sperm had made ittle or no progress toward actually entering the egg mass until after cortical ranule breakdown had covered over three quarters of the egg surface or ntil about 35 seconds after sperm attachment. This result is in contradiction o the account of Just [13]. No attempt was made to follow the later progress f sperm penetration in fixed or living material.

The Behavior of Slowly-fertilizing Eggs

It was evident from the fact that the tops of the fertilization rate and cortical eaction curves of *Psammechinus* were not parallel that the slow fertilizing ggs either had a shorter latent period than the rapidly fertilizing eggs, or ere more susceptible to cortical reaction inhibition due to the presence of he detergent. The latter possibility seemed more plausible, since it was also bserved in these experiments in which the curves were not parallel that the umber of partially fertilized eggs [1, 2] increased in the later aliquots. This hypothesis might explain the results obtained in *Z*, Fig. 1). It seems robable that sperm attachment actually occurred in the slow fertilizing ggs sooner than the fertilization rate curve would indicate (Fig. 3), but these ggs did not conduct an extensive enough cortical reaction to be noticed as partially fertilized under low power.

The Effect of Temperature and of Periodate Ions

One pair of experiments was carried out at 20 and at 10 degrees C with he same batch of gametes, in which both latent period and cortical reaction uration were measured (Fig. 3). Q^{10} values for these periods were 2.32 and 1.43 respectively. One other experiment was carried out at 10°, but without a twin experiment at the higher temperature; however, the latent period duration was identical with the first experiment, and the cortical reaction was retarded by a factor of much less than 2, thus confirming the more significant result in the previous experiment.

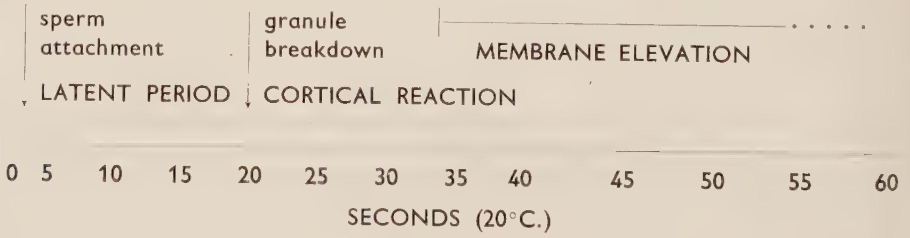
Of serveral chemical treatments applied in an effort to alter the duration of the latent period, only periodate proved effective. Sodium periodate in a concentration of 10^{-5} *M* caused a decrease of about 1.5 to 2 seconds in the latent period: a decrease of 7–10 per cent.

DISCUSSION

The present evidence is not the first to appear indicating a latent period in fertilization. Just [12, 13] realized that membrane elevation did not begin until half a minute after insemination although sperm attached within a second or two of insemination. He claimed to see a "turbidity" change in the cortex in the first 20 seconds (unconfirmed) and a subsequent "wave" that swept over the surface of the egg "clearing" the turbidity. Although the observations have not been repeated, and although Just was not aware of the cortical granules or of their dissolution at fertilization [11], his "wave" began at about the right time for the cortical reaction. Moser, who "rediscovered" the cortical granules and their breakdown at fertilization, stated that breakdown began between ten and twenty seconds after insemination [15]. Rothschild and Swann, in investigating the polyspermy-inducing effect of nicotine, noted in ciné records of 84 eggs that none began a dark-field surface change before 20 seconds after insemination [17]. Although they had no way to establish the time of sperm attachment in each of these eggs, they took notice of this apparent latent period, but correctly drew no conclusions as to its reality or significance. Kaeser [14] also noted this in reviewing the data of Rothschild and Swann. It is entirely justifiable that no firm conclusions were reached by earlier investigators about the latent period of fertilization, for proof of the existence of this period and the extent of its duration require the establishment both of the time of sperm attachment and of the time of cortical granule breakdown at the point of sperm entrance. The present investigation has established the presence of a latent period of about 17 seconds by simultaneous measurements by two interruption methods for determining the time of sperm attachment and of cortical granule breakdown in a population of eggs. Fig. 4 shows diagrammatically the time relations of early events after sperm attachment.

The existence of such an extensive latent period is important for two reasons. First, it appears that there is ample time for various chemical events to take place in the cell before the visible surface change, the cortical reaction, occurs. The temperature coefficient of 2.3 for the duration of the latent period

Fig. 4.—A schematic representation of the time sequence of the visible events in the egg following sperm attachment, based on the data shown in Fig. 3.



vould support the hypothesis that one or more chemical reactions constitute he limiting factor in this period, rather than, for example, diffusion processes lone. The latent period is also important in evaluating the mechanism by vhich sea urchin eggs resist polyspermy. If the assumption is made that a igh proportion of the sperm which arrive at the egg surface are physiologially capable of fertilizing the egg, it is then necessary to assume some mechnism for the exclusion of all spermatozoa except one. Some spermatozoa nay be eliminated by immaturity or other physiological deficiencies; others nay be excluded by mechanical and physiological action of the jelly, acording to Hagström [5, 6, 7]. The cortical reaction and its consequence, the levelopment of the hyaline layer, present a definite structural block against olyspermy. The hypothesis that this process might be the first protection on he part of the egg against polyspermy was explored and later rejected by othschild and Swann [16, 17, 18] in favor of a partial "rapid (propagated) lock" established by two seconds or less and a slower complete block. The xistence of such a rapid block was later disputed [3, 8] on the grounds that partially fertilized eggs (obtained by lauryl-sulfate inhibition of refertilization nder conditions favorable to polyspermy) were found to be capable of efertilization on parts of their surface unaffected by the cortical reaction see also [10]). Since a block taking two seconds or less would have rendered uch refertilization of partially fertilized eggs impossible, it was concluded hat there was evidence for denying the existence of a propagated block more rapid than the cortical reaction, unless such a block were temporary and/or reversed by heat. In the light of present evidence it seems very unlikely that he egg could resist polyspermy without some mechanism more rapid than he visible cortical reaction; an egg which accepts the fertilizing spermatozoon t, e.g. two seconds after insemination would otherwise remain vulnerable or about 17 seconds before the beginning of the cortical reaction. That sea rchin eggs rarely do show polyspermy to an appreciable extent except on ross over-insemination or under experimental treatment might be due to an nknown extent to at least the following three and possibly other factors: (1) he number of sperm reaching the egg in fertilizable condition may decrease vith time due to interaction with the jelly [5, 6, 7]; (2) the egg may possess a high hreshold to stimulation by sperm; this threshold may even vary in different arts of the egg (as it does in the case of electrical stimulation, cf. [4]); (3) he egg may have a propagating block faster than the cortical reaction, as uggested by Rothschild and Swann [18], although this would have to be emporary or reversed by heat, stretching and nicotine [3, 8]. Some of these actors are at present under investigation, and a report will appear in the near future. It is sufficient to point out here that the unexpectedly long latent period found in the present study imposes new requirements on the polyspermy-preventing mechanism in the fertilization of sea urchin eggs.

The data presented here for the duration of the cortical reaction are in substantial agreement with those of Rothschild and Swann, which were derived from cinematographic records of a few equatorially fertilized eggs under dark-field illumination. It is probably reasonable to assume that 20 seconds is the approximate average conduction time for the wave of cortical granule breakdown and surface color change. This agrees well with the best data of Rothschild and Swann [17]; however, it should be kept in mind that considerable variation was encountered among different lots of eggs, and even among different eggs in one batch. The temperature coefficient of about 1.4 for the duration of the cortical reaction suggests that in ripe material diffusion processes may well be the limiting process, rather than chemical reactions. The great spread in conduction times in another experiment carried out at 10 degrees suggests that in eggs with a long conduction time processes other than diffusion may be limiting. Unfortunately the temperature data were not so illuminating as they might have been if rates of conduction had been measured. Attempts to obtain such data were frustrated by the flattening of the egg under the elevated membrane, which made the measurement of the amount of surface affected by the cortical reaction at different times impossible. Such measurements with the present method are not out of the question for the future; all that is needed is a way to let the colloids out of the perivitelline space.

SUMMARY

1. The existence and duration of a latent period of fertilization has been established by a double interruption method. The Hagström method for determining fertilization rate has been used to ascertain the time of successful sperm attachment in a population of eggs. Eggs from the same population were fixed in formalin to determine when cortical granule breakdown began and ended.

2. A latent period of about 17 seconds (12–22 seconds) before the beginning of the cortical reaction was found in eggs of *Psammechinus* fertilized at 20°C. Values of 17–23 seconds were found for the egg of *Arbacia* at 22°C. Duration of the cortical reaction was about 20 seconds in two experiments and 24.5–26 seconds in a third with *Psammechinus* eggs.

3. Temperature coefficients for the latent period suggested that processes

occurring during this time were limited by chemical reactions, while the conduction of the cortical reaction (granule breakdown) is very likely limited by diffusion.

4. Sodium periodate (10^{-5} M) caused a 7–10 per cent decrease in the duration of the latent period.

5. Penetration of the spermatozoon apparently takes longer than previously believed. Sperm heads had only partially penetrated in eggs fixed about 35 seconds after sperm attachment.

6. The extensive latent period found requires that the egg have some polyspermy-preventing mechanism more rapid than the cortical reaction; some possibilities are discussed.

It is a pleasure to acknowledge the hospitality of the officials of the Kristineberg Zoological Station, Dr. Gunnar Gustafson and Professor John Runnström, who provided excellent living material and splendid working conditions for one of us (R. D. A.) during the past summer.

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INTERFEROMETRIC DETERMINATIONS OF THE RIBOSE NUCLEIC ACID CONCENTRATION IN LIVER NUCLEOLI OF PROTEIN-FED AND PROTEIN-DEPRIVED RATS

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Received November 10, 1957

The work of Brachet [7], Caspersson [10] and others has shown the importance of the nucleolus for nucleic acid and protein synthesis within the cell. During such synthesis it is believed to increase in size and ribose nucleic acid (RNA) content.

Stowell [47] observed that the nucleoli in liver cells of rats fed on a low protein diet for periods of 54 and 92 days were larger than those in rats fed on a high protein diet. The present author [38, 41–43] showed that rats starved for 5 days and subsequently fed on a protein-free diet developed large liver nucleoli within three days. Mortreuil and Zajdela [26] found an increase in the nucleolar volume of liver cells after 4 days in rats fed on a protein-free diet. The latter workers gave no measurements or other experimental details. These results appear contradictory when one recalls that protein deprived animals are thought to have a decreased liver protein synthesis. Accordingly an investigation was planned to resolve the conflicting evidence by determination of the RNA concentration in the enlarged nucleoli of liver cells in protein starved rats compared with a control group of animals given a high protein diet. The technique used for RNA estimation was an interferometric determination of the nucleolar dry matter before and after digestion with ribonuclease.

DISCUSSION OF METHODS

The nucleolar dry matter was measured before and after digestion with ribonuclease. That necessitates a fixative that dissolves neither nucleic acids nor, so far as is possible, other substances in the nucleoli. Little information is available on this topic. Carnoy's fluid is said not to dissolve nucleic acids from fresh liver slices during fixation for short periods of time at room temperature [19, 36]. Harbers and Neumann [18], however, found a 20 per cent loss in RNA after 2 hours' fixation. Proteins, polypeptides and amino acids are lost in amounts not exceeding 1 per cent of the total amount of the fresh liver slice [19, 21]. Carnoy's fluid has been successfully used by the Caspersson school in ultraviolet absorption measurements of nucleic acids and proteins. Fixation in Carnoy's solution, however, for longer periods results in a considerable loss of nucleic acids [18, 19, 30, 36, 48, 49].

Fat is dissolved by Carnoy's fluid and lipoidal substances have been demonstrated in nucleoli by histochemical methods. Fautrez and Fautrez-Firlefyn [16] have recently reported on the occurrence of nucleolar phospholipides. On the other hand, Vincent [54], who gives references on this topic, was unable to find significant amounts of fat by chemical methods in isolated starfish oocyte nucleoli. It would appear therefore that the concentration of fatty substances in nucleoli is not sufficiently high to rise serious objection to the use of Carnoy's fluid for nucleolar dry matter determinations. Carbohydrate has not been demonstrated in nucleoli during normal conditions.

Nucleotides of low molecular weight amount to almost one tenth of the total nucleotides of the liver [17]. Their concentration in liver nucleoli is not known. They may partly dissolve during fixation [14, 23, 24]. If they dissolve during ribonuclease digestion they may interfere with the RNA determination. Accordingly, such low molecular nucleotides were extracted from a number of sections by the method of Edström [14]. The nucleolar dry matter was determined before and after extraction and no significant loss was demonstrated. On this evidence, extraction was abandoned, in order to minimize the handling of the sections.

RNA is completely dissolved by buffered ribonuclease solution from Carnoy-fixed liver, spinal ganglia [14] and pancreas [23, 24]. This solvent action is thought to be highly specific. No proteins are dissolved [14, 23, 24]. Of other soluble substances, desoxyribose nucleic acid (DNA) is not present in liver nucleoli [22] and diffusible, low molecular nucleotides have already been eliminated as a possible cause of error. t is not known to what extent inorganic compounds remaining in the nucleoli after fixation dissolve during treatment with ribonuclease. It is unlikely that they form a significant proportion of the fixed nucleoli. Thus the dry matter dissolved can be regarded as RNA. The concentration of RNA in the nucleoli can be expressed as a percentage by dry matter estimations performed before and after ribonuclease digestion. This figure is relative rather than absolute, as some substances are dissolved during pretreatment (see above). No other fixative is known to be superior to Carnoy's luid for the purpose.

It is well known that a redistribution of substances may occur during chemical fixation. Accordingly an attempt was made to use freeze-dried slices. Sections cut from such slices must be fixed to permit selective enzymatic digestion. Otherwise undenaturated substances may dissolve in the buffer solution. Freeze-dried sections, fter-fixed in Carnoy's solution for one hour, passed through a control buffer solution nd finally stained with gallocyaninchromalum showed considerable distortion of the tructure. Freeze-dried and after-fixed slices not treated with buffer solution did not how such distortion. There are several possible explanations for this phenomenon. The denaturating effect of Carnoy's solution may be less on the water-free freeze-dried section than on fresh tissue and such undenaturated substances may dissolve in the buffer. Several enzymes are known to remain in an active state in the freeze-dried section and may act on constituents during incubation in the buffer [4, 5]. In any event the use of freeze-dried slices after-fixed in Carnoy's solution was abandoned on account of the distortion effect.

The percentage of nucleolar RNA was calculated by determination of the nucleolar ry matter before and after digestion with ribonuclease. Such a method necessitates knowledge of the refractive indices of the nucleoli before and after extraction. That was determined in the following way. The phase change caused by a nucleolus in two different media, namely glycerol and liquid paraffin, was determined before and after digestion with ribonuclease. The refractive indices were then calculated by the formulae [3]

$$\phi_1 = (n - n_1) \cdot t \quad \text{and} \quad \phi_2 = (n - n_2) \cdot t$$

$$\therefore \quad n = \frac{n_2 \cdot \phi_1 - n_1 \cdot \phi_2}{\phi_1 - \phi_2}$$

where n = the refractive index of the nucleolus; n_1 = the refractive index of glycerol (1.451) (as measured at 20°C 546 mμ with a Leitz microrefractometer II "Jelly"); n_2 = the refractive index of liquid paraffin (1.477); ϕ_1 = the optical path difference between nucleolus and interference bands in glycerol; ϕ_2 = the optical path difference between nucleolus and interference bands in liquid paraffin, and t = the thickness of the nucleolus.

The refractive index was found to be 1.557 before and 1.550 after digestion with ribonuclease. There is no statistical difference between these two figures that are founded on twenty measurements.

The percentage of nucleolar RNA can therefore be calculated from the formula

$$\frac{x \cdot \phi_3}{100} = \phi_3 - \phi_4,$$

where x = the percentage of nucleolar RNA; ϕ_3 = the optical path difference between nucleolus and interference bands in any medium, e.g. liquid paraffin, before digestion with ribonuclease, and ϕ_4 = the optical path difference between nucleolus and interference bands in the same medium after digestion with ribonuclease.

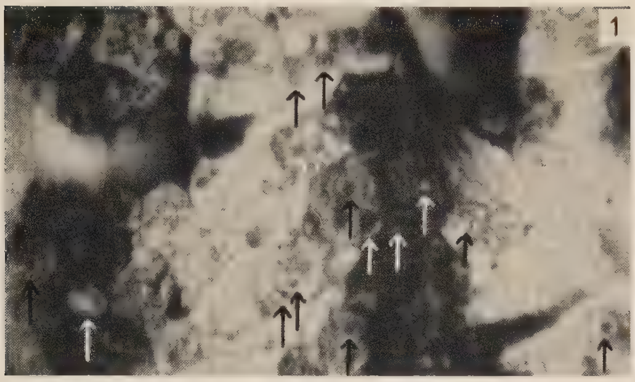
MATERIAL AND METHODS

Male rats (*Mus norw. alb.*) of a strain over a long period bred by the author, were employed. They were reared on mouse bread (Konsums bakeries, Stockholm), water and whole milk. Sixteen animals with an individual weight of about 200 g were chosen for the experiments and were starved for 5 days. Eight of them were subsequently fed on a 25 per cent casein diet and the remainder on a protein-free diet *ad libitum* [41] for 3 days when they were sacrificed. All animals were killed between 3 and 3.30 p.m. The animals were kept in wooden cages with saw dust on the floor.

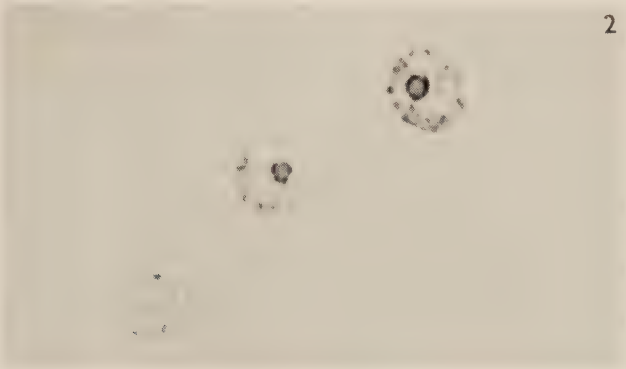
Liver slices were fixed in Susa for volumetric determinations of nucleoli by a previously described technique [41, 43].

Other slices were fixed in Carnoy's solution for 1½ hours for determination of the concentration of RNA in the nucleoli. Nucleic acids were extracted from the evaporated fixative by the method of Ogur and Rosen [32] but could not be demonstrated in significant amounts by ultraviolet absorption measurements. Accordingly, no appreciable losses of nucleic acids can have occurred during fixation.

The slices were passed through absolute alcohol and embedded in paraffin. Sections were cut with a piece of Scotch tape glued to the paraffin block and the microtome



ig. 1.—Section of liver from a protein-deprived rat. A few nucleoli are indicated by arrows. Microhotograph with interference bands in the field. For technical details see text. × 570.



ig. 2.—Section of liver from a protein-fed animal after ribonuclease digestion and gallocyaninhromalum staining at pH 1.0. Only DNA-containing structures are stained. The nucleoli are ardly stained but are surrounded by DNA-containing chromatin. No cytoplasmic structures are tained. × 1500. N.A. 1.32.

et at zero to secure thin sections [15, 35]. The sections were placed on albumenized lides without employment of any water. The tape was removed by immersing the lides in xylene overnight. They were mounted in liquid paraffin and examined under a Dyson interference microscope (Cooke, Troughton & Simms, England). Nucleoli at the periphery of the sections were chosen at random, focused and photographed with nterference bands in the field (Fig. 1). A 250 watt compact mercury arc lamp (British Thomson Houston) with an Ilford mercury green filter 807 was employed to provide monochromatic light, 546 mμ. The optical system comprised a 95 × immersion objective N.A. 1.3 and a 6 × compensation ocular. The linear magnification on the photographic plates (Gevaert orthochromatic, 9 × 12) was 570 ×.

The sections were taken through xylene and alcohols to water and digested in a solution containing 5 mg protease-free ribonuclease (2 × crystallized, Worthington

Biochemical Sales Co.) per 100 ml for 3 hours at 37°C in McIlvaine's buffer at pH 7.0 [37, 39]. The slides were rinsed with water and brought through alcohols and xylene to liquid paraffin. The same nucleoli were again photographed under the interference microscope. The sections were finally stained with gallocyanin-chromalum at pH 1.0 for cytological control of the ribonuclease digestion. This technique seems to be specific in staining nucleic acids in liver cells (Fig. 2) [37, 39, 40].

The blackness of nucleoli and interference bands on the photographic plates were registered on photographic paper by a recording microdensitometer along lines perpendicular to the interference bands, the light cone being adjusted to cover as much as possible of the nucleolus. The optical path difference between interference bands and the centres of the nucleoli was measured. The distance between two dark, adjacent bands on the photographic paper was 283 mm.

Examination of the sections under the interference microscope in white light revealed that the path difference caused by the nucleoli varied between $\frac{1}{9}$ and $\frac{1}{3}$ λ and did not exceed one λ. By using very thin sections interference from nuclear sap or other substances above or beneath the nucleolus should have been insignificant and calculations were made on this assumption. No substantial error can have arisen from this source, as the path difference caused by nuclear sap alongside to the nucleolus was always very small or none.

The experimental error for individual observations was determined by photographing the same nucleolus in various locations within the interference field. The error, estimated as the standard deviation, was $< \pm 0.015\ \lambda$. Six nucleoli from each animal were photographed. Each nucleolus was photographed twice before and twice after the ribonuclease digestion. Some of the plates had to be discarded as the nucleoli were not precisely in focus or could not be refound. The percentage of RNA was calculated for each nucleolus by taking the mean of the figures for optical path difference. The median figure for RNA concentration was calculated for each animal. The mean of the two median figures was used for odd numbers of nucleoli. For each group of animals the mean, the standard error and the standard deviation of the mean were computed from the medians of the individual animals. The t-test was used to establish possible statistical differences between the groups.

RESULTS

The results are summarized in Table I. Statistical analysis revealed a significantly larger nucleolar volume in the case of protein-deprived animals as compared with protein-fed animals ($P < 0.001$). There was no difference between the groups in concentration of RNA in liver nucleoli.

Fig. 1 shows a section from the liver of a protein-deprived animal and photographed with interference bands in the field. Representative nucleoli are indicated by arrows. Fig. 2 shows a liver section from a protein-fed animal after ribonuclease digestion and staining with gallocyanin-chromalum at pH 1.0. Only structures containing DNA are stained and the appearance is of dark staining chromatin rings at the periphery of the faintly stained nucleoli.

TABLE I.

Diet	Nucleolar size in μ^3			Nucleolar RNA in per cent of nucleolar dry matter	
		s	n	%	n
25 % casein	0.9±0.06	0.5	80	13	5
	1.1±0.10	0.9	80	17	5
	1.2±0.08	0.7	80	12	3
	1.1±0.08	0.7	80	18	5
	0.9±0.05	0.5	80	15	6
	1.0±0.07	0.6	80	17	6
	1.0±0.07	0.6	80	15	4
	1.0±0.06	0.5	80	8	6
	Σ 1.0±0.04	0.1		Σ 14±1; $s=3$.	
Non-protein	1.5±0.10	0.9	80	20	6
	2.1±0.17	1.4	80	14	5
	2.2±0.18	1.6	80	17	7
	2.2±0.22	2.0	80	11	3
	1.6±0.12	1.1	80	13	5
	1.4±0.09	0.8	80	12	3
	2.0±0.15	1.4	80	12	4
	1.6±0.12	1.1	80	13	5
	Σ 1.8±0.12	0.3***		Σ 14±1; $s=3$.	

*** Signifies a statistical difference of ($P<0.001$) between the groups.
s = the standard deviation of the mean; n = the number of nucleoli examined in each animal.

DISCUSSION

As briefly mentioned in the introduction Stowell [47] performed experiments similar to those here reported. Many of his rats fed for long periods on low protein diets (containing 5 per cent brewer's yeast) died of infection during the experiment. Vitamin deficiency, however, cannot be excluded as a possible factor. In these experiments rats fed on the protein deficient diet for 54 or 92 days developed larger liver nucleoli than protein fed animals and measurements with the ultraviolet microscope showed the nucleic acid/protein ratio to be the same for the nucleoli of either group. This ratio for liver cytoplasm was also the same in both groups. Stowell, therefore, concluded that the liver cells of protein-depleted rats do not produce large amounts of cellular protein substances. Unfortunately, no information is given as to the amount of nucleic acid or protein in relation to the size of the nucleolus.

Few quantititative studies of nucleolar nucleic acids have been reported.

Caspersson and his co-workers have made ultraviolet absorption measurements mainly on freeze-dried but also on Carnoy-fixed sections. For the most part no absolute figures are given. They reported a nucleolar concentration of RNA of up to 7 per cent of the nucleolar mass in the cells of several tissues obtained from a large number of species ([10, 11, 20, 31, 50] and others). Nurnberger [30 a] found about 3 per cent RNA in rat liver nucleoli. The protein concentration is reported as falling within the range 20 to 40 per cent in most cases [10].

Dounce *et al.* [13, 25] have studied the composition of isolated liver cell nucleoli. They found that DNA and RNA respectively represent 12–18 per cent and 1–4 per cent of the dry weight. Histological studies indicate that there is no DNA in rat liver nucleoli [22], although they are surrounded by a ring of Feulgen-positive material which may have contaminated the nucleoli in Dounce's experiments. Such material probably originates from chromosome threads. It is therefore possible that the figure obtained for nucleolar RNA concentration in this study is too low.

There are two reports of estimations performed on starfish oocyte nucleoli. Vincent [53] reports RNA values ranging from 2.2 to 4.6 per cent of the dry weight of nucleoli isolated in distilled water, and in a later paper [54] values as high as 8 per cent in immature oocytes down to 1.7 per cent in mature oocytes. These results were obtained by measurement of ultraviolet absorption of nucleoli both in isolation and *in situ*. The variation in concentration is at least in part attributed to the presence of large vacuoles in the nucleoli of mature oocytes. Baltus [1, 2] who isolated starfish oocyte nucleoli in sucrose solution found a RNA concentration of 5 per cent of the dry weight. It is not known to what extent RNA may dissolve from nucleoli during the isolation procedure.

Pollister and Leuchtenberger [33] examined the nucleoli of Carnoy-fixed sporocytes of *Zea* mays and found that 53 per cent of the substance was removed as measured by the extinction at 2537 Å. That has been estimated to correspond to 7 per cent on a dry matter basis [13]. About one fourth of the dissolved substances are claimed to be a polymer which is intermediate between nucleic acid and mononucleotide.

The present author found a RNA concentration of 14 per cent of the dry weight in rat liver nucleoli. Because some low-molecular substances were certainly dissolved during the histotechnical treatment this figure may be slightly higher than the true value. The dry matter has previously been found to represent 37–38 per cent of the total nucleolar mass [44], and therefore the concentration of RNA in the material studied can be expressed as 5 per ent of the total nucleolar mass. This figure corresponds closely with those btained by the Caspersson school for other mammalian cells.

The demonstration of equal proportions of dry weight and RNA in the arge liver nucleoli of protein-deprived rats on the one hand and the small iver nucleoli of protein-fed rats on the other indicates an absolute increase f RNA and dry matter in the former.

As mentioned in the introduction large nucleoli with increased amounts f nucleic acids are generally thought to indicate an increased nucleic acid nd protein synthesis in the cell. Numerous investigations have demonstrated decrease in the liver and plasma protein values in rats deprived of dietary rotein. It is therefore generally assumed that protein synthesis in the liver f a protein deprived animal is decreased. In addition investigations using iver slices from protein deprived rats have revealed a decrease in tissue proein synthesis [6, 34]. The liver nucleic acids behave differently. In rats fed n a protein-free diet, despite a reduction in the total amount of liver RNA here is an increased turnover of the residue so that the total actual synthesis f RNA is equal to or larger than that present in protein-fed rats [8, 9, 12, 28, 29, 52]. This is true for all parts of the cell, including the nucleus. The RNA f the microsomes, thought to correspond to the basophilic inclusions of ixed cells (for a review on this topic see Stenram [45]) and especially conerned with protein synthesis [7], behaves in the same way [27, 51]. The RNA f the liver cell is thus metabolically active in the protein-deprived rat. The resent investigation has demonstrated a quantitative increase of nucleolar RNA during protein depletion, and suggests that the enlargement of the liver ucleoli indicates an intense nucleic acid metabolism. The reason for this ctivity is not known. It may be due to the absence of certain essential amino cids from the diet as large liver nucleoli have been demonstrated in animals ed on a diet otherwise adequate but devoid of any one of methionine, lysine, soleucine, threonine, tryptophane or valine. The absence of histidine, leucine r phenylalanine caused no increase in liver nucleolar size [42]. It is not the bsolute deficiency of essential amino acids that causes the increase in nuleolar volume because starved animals have smaller liver nucleoli than animals fed either a protein containing or a protein deficient diet [22, 41]. Certainly the liver nucleoli of protein-fed animals are also metabolically ctive as indicated by their high RNA content and the synthesis of cellular nd plasma proteins by the liver cells.

The increase in nucleolar size during protein depletion seems to occur nly in normal liver tissue. It was not found in exocrine pancreas cells, spinal ord cells (unpublished observations) nor in hepatoma cells [46].

SUMMARY

Liver nucleoli of rats fed on a non-protein diet were considerably larger than those of rats fed on a 25 per cent casein diet. The concentration of ribose nucleic acid (RNA) of the nucleoli in Carnoy-fixed sections was determined by interferometric estimation of the dry matter before and after digestion with ribonuclease. The RNA concentration was 14 per cent of the nucleolar dry matter in both groups, corresponding to 5 per cent of the total nucleolar mass. The results indicate absolute increase of RNA in the large nucleoli of protein-deprived animals in comparison with protein-fed animals. It is concluded that there is intense nucleic acid metabolism in these enlarged nucleoli. The liver nucleoli of protein-fed animals are also thought to be metabolically active.

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Experimental Cell Research **15**, *184–190* (*1958*)

AN APPROACH TO THE PROBLEM OF EMBRYONIC INDUCTION[1]

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Received November 12, 1957

Today there are two different trends of thought regarding the nature of the factors causing differentiation, or at least regarding the so-called Spemann organizer. Many investigators look upon the effect of the organizer on the ectoderm as unspecific or as a kind of "evocation" or "activation" [1, 8, 18]. This view is corroborated by Holtfreter's investigations on the effect of cytolyzing factors on totipotent ectoderm [4, 5]. On the other hand, the results of numerous experiments with so-called heterogeneous inductors prepared from adult foreign tissues seem to indicate that neural induction is caused by a chemical substance or group of substances [16, 14, 6]. Even more clearly has the influence of specific factors been revealed in the process leading to the differentiation of totipotent ectoderm into mesoderm. Toivonen's finding [12] that the guinea-pig bone marrow is an almost pure mesodermal inductor afforded the possibility for more accurate chemical determination of this factor. Toivonen [13] found that the mesodermal inductor in the guinea-pig bone marrow is thermolabile, like the proteins. When Toivonen and Saxén [17] simultaneously used guinea-pig liver as neural inductor and guinea-pig bone marrow as mesodermal inductor, all regional types of differentiation, both neural and mesodermal, were induced. On fractionation and treatment of guinea-pig bone marrow with ethanol and butanol, Kuusi [7] observed that the mesodermal inductor in this tissue behaved like the proteins. These results constitute evidence in favour of the theory that the mesodermal inductor is a specific chemical substance or group of substances.

In addition, this view is corroborated by the observation of Niu and Twitty [10] and Niu [9] that it is possible to extract from chordamesoderm with saline a factor, or factors, which induce both neural and mesodermal changes when a totipotent ectoderm is placed in this solution. Furthermore, Niu found that the inductive agents are not dialyzable and that their spectrum resembles that of the nucleoproteins. Even when enzymatic treatment of the nductive agents diffusing from the chordamesoderm gave converse results, t was obvious that a chemical agent of macromolecular type is involved.

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[1] This investigation has been reported at greater length in *Ann. Acad. Sci. Fennicae A* **35** (1957), where the preparation and testing of the immune sera is described in detail.

The object of the present investigation was to study how the *Triturus vulgaris* gastrula reacts to implants of guinea-pig liver, kidney and bone marrow tissue treated with homologous or heterologous rabbit immune sera. n other words, the question posed was whether antiserum inhibits or alters the inductive capacity of the tissue.

MATERIAL AND METHODS

Guinea-pig liver, kidney and bone marrow were used as inductor material. The inductive effect of these tissues on the ectoderm of the *Triturus vulgaris* gastrula is known [12, 15]. Before implantation, the tissue pieces were treated as follows: (1) Immersion for half an hour at +37°C and overnight at +4°C in one of the antisera or in normal rabbit serum; (2) rinsing with Holtfreter solution; (3) immersion in 70 per cent ethanol for 3 hours at +4°C; and (4) repeated rinses in Holtfreter solution.

After this treatment the tissue piece is implanted into the blastocoele of young *Triturus* gastrula.

The antisera were prepared in rabbits, the saline- or phosphate buffer-soluble fraction of guinea-pig liver, kidney or bone marrow being used as antigen. After two series of intravenous and one series of intraperitoneal injections, the activity of the antisera formed was determined against homologous and heterologous antigen, by the interface and gel diffusion precipitation techniques, guinea-pig red cell agglutination, and absorption of the antisera. The antisera were found to be of high titre and relatively organ-specific.

Implantation of the tissue pieces was performed in the manner previously described [15]. The tadpoles were fixed in Bouin's picroformol 11 13 days after implantation, stained *in toto* with boraxcarmine, serially sectioned, and counter-stained with Blauschwartz.

RESULTS

In the evaluation of the results those tadpoles were considered in which either the implant, or inductions, or both were microscopically discernible. Cases in which the implant was observable under the ventral epidermis without inductions were regarded as "inhibited", whilst those cases in which inductions were observed in addition to the implant were regarded as "uninhibited". The material is accordingly presented in Fig 1. It is seen that in almost every series where immune sera have been used, cases occurred in which the implant was observable under the ventral epidermis without inductions. The largest number of such cases are found when guinea-pig bone marrow was treated with various antisera. Homologous antiserum

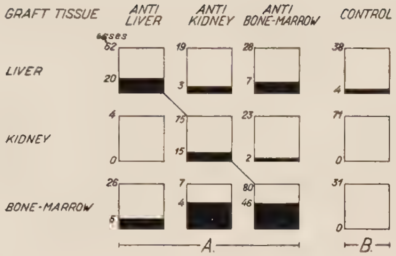

Fig. 1.—Summary representation of implanted cases. *The black area* shows the percentage of inductor-positive and induction-negative cases. (*A*) Homologous or heterologous anti-guinea-pig organ sera have been used; (*B*) normal rabbit sera have been used for the sake of controls. Implant tissue: guinea-pig liver, kidney or bone marrow. The series in which the implants were treated with homologous antisera are connected by lines.

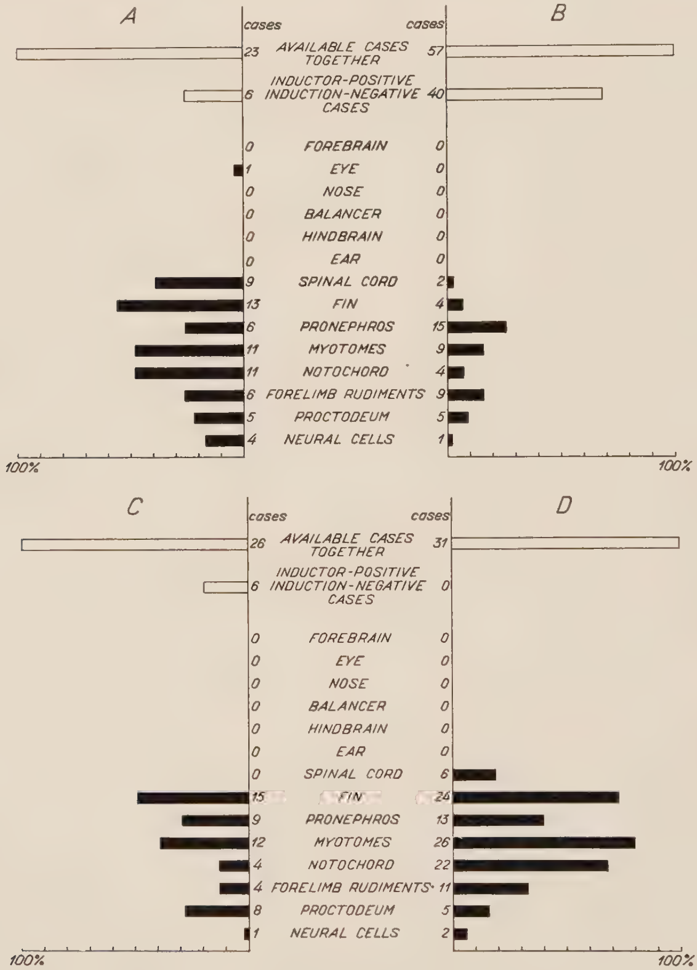

Fig. 2.—Percentage distribution of inductor-positive and induction-negative cases and of inductions in the implantation series where guinea-pig bone marrow implants were treated with: (*A*) antibone marrow (saline fraction); (*B*) anti-bone marrow (phosphate buffer fraction); (*C*) anti-liver; and (*D*) normal rabbit sera.

seems to have the strongest effect, the inductor being "inhibited" in over 50 per cent of cases in this series. In the corresponding kidney series, some "inhibited" cases occurred, whereas no such cases were observed in the series where the kidney implants had been treated with normal rabbit serum.

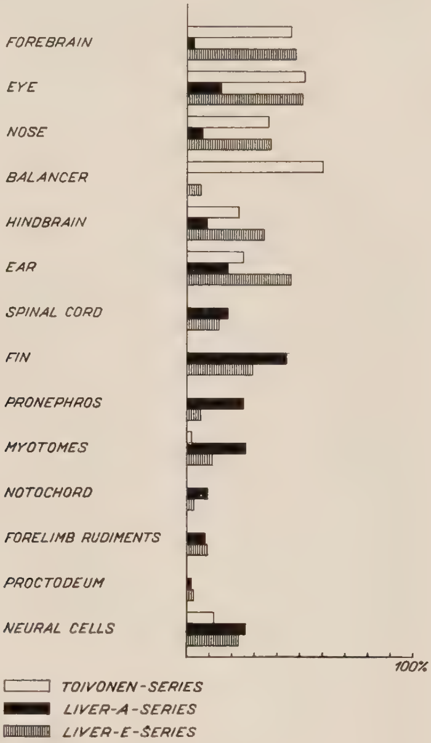

Fig. 3.—Comparative representation of the qualitative distribution of the inductions caused by guinea-pig liver implants treated with anti-liver (saline fraction) serum (A-series), with normal rabbit serum (E-series) and in a series published by Toivonen and Saxén [17] including 113 cases.

In the liver implantation series, too, many cases occurred in which the implant may be regarded as "inhibited". These were found not only in the immune serum series, however, but also in the control series.

On closer examination of the bone marrow implantation series in which the bone marrow tissue had been treated with some immune serum, it is found that in the induction-positive cases the inductions were mostly mesodermal. Notochord and myotomes were frequent findings. In some cases spinal cord was also observed. A mesodermal induction picture was also seen in all cases where the implant had been treated with normal rabbit serum (Fig. 2).

In the liver implantation series "inhibited" cases were also observed after treatment with normal rabbit serum (Fig. 1). In the cases exhibiting inductions, spinocaudal formations occurred in abundance in addition to archencephalic and deuterencephalic structures. Fig. 3 shows the qualitative distribution of the inductions in the guinea-pig liver implantation series, where the implants had been treated either with rabbit antiserum prepared against the saline-soluble fraction of the liver, or with normal rabbit serum. In addition, the qualitative distribution of the inductions in a similar series reported by Toivonen and Saxén [17] is presented in Fig. 3.

DISCUSSION

It appears from this brief survey of the results that it is possible to influence the action of the inductive agent of various guinea-pig organ tissues with rabbit immune sera. It is difficult, however, to define the way in which homologous or heterologous antisera acted on the tissue pieces *in vitro* in these cases. In the present experiments the antisera were not absorbed, and were not inactivated. It may be assumed that, in addition to species-specific antibodies, they also contained organ-specific [3] and heterospecific [20] antibodies, in which precipitating, agglutinating and lytic components were represented. Furthermore, it is possible that the incomplete forms of the antibodies prepared played an essential part in the effect of the antisera. This would imply that antibody globulins are attached to the surface of the cells of the tissue pieces, influencing not only the reactive groups of the cell surface but also its permeability. It is obvious, however, that no conclusions can be drawn from the present results with regard to the character of the factors causing inhibition of the inductive action of the tissues.

In this study only those cases were included in the material in which implant, induction or both were discernible. A number of cases were discarded which in microscopical examination did not exhibit any of these features. In some of these, the implant had probably remained *in situ* long enough to cause induction, but was "inhibited", whilst in other cases it had immediately slipped out of the incision without having had time to exert an effect. In some cases, particularly in the bone marrow series, the implant was resorbed. Owing to these discarded cases, the distribution presented in Fig. 1 is obviously erroneous, the percentage of "inhibited" cases being, in fact, higher.

It was a feature of the technique that the tissue pices were treated, where possible, when already cut to the size that was suitable for implantation. During implantation it was necessary, however, to handle the pieces with instruments, and obviously they were thus torn in some cases. Hence, it is possible that in the implants consisting of the largest pieces there were areas of the surface on which the antiserum had not acted. This possibly explains the fact that in spite of treatment with immune sera, cases also occurred in which the induction picture was the same as in the controls. The inductive action of the tissue pieces was thus either completely "inhibited" or "normal", depending on whether the antiserum had acted on the whole tissue surface or not. This assumption suggests the possibility that the cell surfaces of the inductive tissues are of significance in the process of induction [19].

In the immune serum series, it was the inductive action of the guinea-pig bone marrow tissue that was most strongly "inhibited", whilst the inductive action of the kidney and liver tissues was influenced in a smaller number of cases. As mentioned in the foregoing, it has been suggested that two qualitatively different groups of factors are active in the early stage of the "natural" process of differentiation during gastrulation, and that the resulting induction picture is dependent on the amount and ratio of these factors [17]. The protein character of the mesodermal factors has been repeatedly referred to, whilst the neural factors have proved to be less specific. This may perhaps explain the fact that the bone marrow inductor, which contains mesodermal factors in abundance, is more readily "inhibited" with immune serum than the liver and kidney inductors, which contain a rich supply of neural factors.

In many previous investigations the guinea-pig liver has proved to be an almost pure archenecephalic inductor [17, 15]. Nonetheless, after treatment with rabbit serum the liver tissue was found to induce both deuterencephalic and spinocaudal structures in even greater amount than archencephalic formations (Fig. 3). This was observed both in the immune serum series and in the series treated with normal rabbit serum. On the other hand, the liver tissue has proved to be a weak inductor [2], often observed after implantation under the ventral epidermis of the host without inductions. It is possible that the change in the mesodermal direction that was observed in the inductive action of the liver tissue was due to the treatment with rabbit serum. Later it has proved possible to demonstrate factors in the guinea-pig serum which induce mesodermal structures in implantation experiments [11]. The inductive action of the serum is still under investigation.

As pointed out, the present results are still preliminary, inasmuch as unfractionated tissues and their antisera were used. Hence it was not possible to determine the nature of the antibody effect. The study is to be continued with tissue fraction containing only known antigens in the smallest possible number. Preliminary investigations on tissue culture cells and serum fractions have been performed. On the basis of the present study it may be assumed, however, that the technique employed affords possibilities for investigating the nature of the factors that are active in the process of differentiation.

SUMMARY

The effects of rabbit immune sera in the inductive capacity of guinea-pig liver, kidney and bone marrow tissue was studied in implantation experiments with *Triturus vulgaris* gastrulae as host animals. In the preparation of the immune sera the saline- and phosphate buffer-soluble fractions of guinea-pig liver, kidney and bone marrow were used as antigen material.

It was found that the antisera inhibited the inductive action of the bone marrow tissue, which was in contrast to the result with normal rabbit serum. The inductive action of the kidney and the liver was also influenced by the antisera, although the inhibitory effect was indistinct.

The inductive effect of the guinea-pig liver tissue, which in many previous investigations has proved to be almost purely archencephalic, changes in the mesodermal direction after treatment with rabbit serum. It is suggested that the factor causing mesodermal differentiation was adsorbed from the rabbit serum onto the liver tissue.

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xperimental Cell Research 15, 191–199 (1958)

INTERACTION BETWEEN Ca OR Mg IONS AND NUCLEOPROTEIN FROM SEA URCHIN SPERMS

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Received November 20, 1957

The purpose of this investigation was to give a more quantitative picture f the interaction between Ca or Mg ions and deoxyribonucleoprotein (DNP) han hitherto published. This interaction has been noted earlier in a quali- ative way in the strong precipitating effect of Ca or Mg ions on DNP, by ilbert *et al.* [6], Luck *et al.* [10], and by Frick [5] among others.

The investigation was partly inspired by a hypothesis suggested by Mazia 11] that Ca ions play an essential part in the structure of the chromosomes hrough the formation of links between subunits of macromolecular com- lexes of protein and nucleic acid. Mazia was first led to this idea when ernstein and he [3] observed that the material from sea urchin sperms ontaning DNP had to be treated with citrate in order to bring the DNP nto solution. Mazia [11] later made a cytological study of the effect of chelating gents on the chromosomes from *Drosophila* and from grasshopper testis, hich led to the suggestion of the hypothesis just mentioned.

In a recent cytological study Kaufmann and McDonald [9] confirmed the trong effect of chelating agents on chromosomes, but criticized the detailed tructural concepts of Mazia. Davison and Butler [4] have denied the pos- ibility of the hypothesis on stoichiometric grounds, as they found only 0.05 er cent Ca in their DNP preparations from calf thymus. However, differ- nces in source and preparation make a comparison uncertain. Apart from he role that Ca or Mg ions may play in the chromosomes *in vivo*, the effect f the addition of salts containing these ions is of interest *per se*. The methods ow in use to isolate whole nuclei according to Schneider and coworkers 13, 8] or according to Philpot and Stainer [12] prescribe the addition of a or Mg ions, respectively. For refined physical-chemical measurements n DNP, the question of the effect of the quantities of Ca and Mg present as ontaminants is also of importance.

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PROCEDURE

All work was done in the cold, i.e. around + 5.5°C.

The sperms from sea urchin (*Strongylocentrotus purpuratus*) were prepared as described by Bernstein and Mazia [2] and washed twice in ice cold sea water. A sample was then put under the microscope to make sure that the sperms looked healthy and lively. They were then washed once more with sea water and centrifuged down in an ordinary laboratory centrifuge (1200 × *g*) for 15 minutes. The same time and speed for centrifugation were used between all washings, unless otherwise noted. The sperms, with a wet weight of about 0.5 g, were washed twice with 50 ml 1/8 *M* Na citrate. The suspensions were then divided equally into two tubes. The sample in each tube was next washed twice with 0.03 *M* NaCl per tube being used for each washing. During the second NaCl washing, the sperms burst, and a white lump of DNP was formed in the tubes, quite distinguishable from the slightly coloured sperm membranes. After this point each of the two tubes was treated separately and differently in the first preparation made in these experiments, but in all subsequent preparations only the procedure given below for tube number 2 was followed.

The lump of protein in *tube number 1* was washed rather rapidly with 25 ml 0.01 $NaCl_2$ to remove the remaining citrate and then put in 100 ml 0.003 *M* $CaCl_2$ for 20 hours.

The protein lump in *tube number 2* was washed with 25 ml 0.03 *M* NaCl and then put in 100 ml 0.009 *M* NaCl for 20 hours (i.e. same ionic strengths for the NaCl as for the $CaCl_2$ solutions).

After 20 hours both lumps were still intact, but the lump in tube 1 had a rather grainy surface, and the supernatant contained traces of a light precipitate, whereas that in tube 2 looked slimy and the supernatant was quite clear. Both lumps were then transferred into a fresh 100 ml 0.003 *M* $CaCl_2$ and 100 ml 0.009 *M* NaCl, respectively. Both samples were then treated in a Waring Blender at high speed for 80–90 seconds, after which the sample in tube 1 (with Ca^{++}) formed a fine grainy suspension, from which the particles settled rapidly, leaving a clear supernatant. In tube 2 (with Na^{+}) the suspension did not settle, and since the viscosity increased, presumably DNP dissolved quite easily, resulting in an opalescent solution.

Both samples were allowed to stand in the cold for 2–4 days. Sample number 1 (Ca^{++}) was shaken by hand from time to time. No change could be observed after 4 days. The two samples were then treated as follows.

Sample from tube 1 (with Ca^{++}*)*

Experimental and Discussion

According to the hypothesis being tested in these experiments, sample number 1 can be looked upon as containing a DNP which has lost its natural content of Ca by treatment with citrate. The addition of Ca ions to this DNP would therefore be responsible for making its solubility differ greatly from that of a parallel sample with no Ca ions added. If this mechanism is actually operating, a minimum demand would be that the added Ca ions should, in turn, be removed by citrate, after which the solubility of the DNP should increase.

One-fifth of the sample was washed three times with 20 ml of $\frac{1}{8}$ M Na citrate. The washings were performed by repeated suctions up and down a pipette. Between the washings the sample was centrifuged as usual. The DNP was then washed in the same way twice with 20 ml 0.05 M NaCl, centrifuged in two tubes and one pellet was put in 20 ml distilled water and one in 20 ml 0.003 M NaCl. The sample changed in appearance even during the washings, from a grainy to a soft, slimy type. Both pellets went rapidly into solution. After 20 hours of standing in the cold the two solutions were very viscous. When centrifuged in the Servall centrifuge at $8000 \times g$ for 15 minutes the gel formed in the sample with 0.003 M NaCl was pressed to the bottom as a voluminous pellet with a clear supernatant. The DNP dissolved in distilled water went half way down the centrifuge tube with the supernatant still containing more than 10 per cent of the UV absorbing material. This gel became stiffer on standing and also easier to centrifuge down. However, if DNAse (Worthington Biochemical Corp. DML 547–8) was added, the gel was completely broken up, resulting in a solution of low viscosity.

The effect of the citrate on added Ca seems to be of the type supposed by Mazia for naturally occurring Ca^{++}, thus strengthening the *possibility* of that hypothesis. As described above, the parallel preparation of DNP in NaCl (tube 2) gave a highly viscous solution. Although a small part of the material in this solution will be brought down by centrifugation at $8000 \times g$, it will not show the same strong tendency to form gels on standing. The difference may serve as a reminder of the strong influence of preparation technique on the behaviour of the DNP molecule in solution.

Sample from tube 2 (with Na^+)

Experimental and Calculation

Aliquots of 10 ml were taken from sample 2 and centrifuged in the Servall for 15 minutes at $8000 \times g$. A very small pellet was formed, mostly of the slightly coloured membrane material. To the supernatant was added 2 ml 1 M NaCl and a white precipitation was immediately formed. After half an hour the precipitate was centrifuged down in the laboratory centrifuge. The supernatant was tested in the Beckman spectrophotometer and it was found to contain less than 1 per cent of the total DNA as judged from the absorption at 260 mμ. Most of the absorption must come from dissolved protein and from unspecific absorption. This is quite in line with the result found by Bernstein and Mazia [2]. The DNP pellets were shaken with 20 ml of $CaCl_2$ of different strengths. The $CaCl_2$ solutions were made up from a 0.100 *M* solution. The chloride was taken from a previously unopened bottle of $CaCl_2$, $2H_2O$, Mallinckrodt 4160. One series of solutions was made from anhydrous $CaCl_2$, Baker code 1510. All dilutions lower than 0.01 *M* were made with double distilled water (freshly made) and the solutions were kept in well washed plastic bottles. The samples of DNP in $CaCl_2$ were allowed to stand 2 days in the cold and were shaken from time to time. The samples were centrifuged in the Servall at 8000 × *g* for 20 minutes (unless otherwise stated). Six-tenths of the supernatant were carefully taken off. The optical density (OD) was then determined in the Beckman spectrophotometer after dilution. The solution was at the same time made 0.1 in NaOH. The absorption at 330–370 mμ was always checked and the OD at 260 and 280 mμ determined. The absorption at 330 and higher was very small in comparison with that at 260 except in experiments with very dilute solutions (see below).

In all, three different preparations of DNP were made and 8 precipitation curves with Ca^{++}. Of these, one is omitted in Table I, as the curve could not be drawn with enough accuracy. The OD values of the curves in Fig. 1c have been obtained after correction according to Heller and Vassy [7] for the background absorption as revealed from the values for 330–370 mμ. This correction makes the curve with the lowest values (number 7) less

TABLE I. *The optical density at 260 mμ at the start of precipitation for different concentrations of DNP from sea urchin sperms in solutions of $CaCl_2$.*

The strength of the salt has been varied and the values given below for optical density of DNP and molarity of salt are at the precipitation point as shown in the curves in Fig. 1. For explanation of the value *b/a* see text.

Curve number	Prep of DNP	OD	Log C_{CaCl_2}	b/a^a
1	III	15.7	−4.22	—
2	I	8.0	−4.15	4
3	III	5.7	−4.16	7
4	III	3.1	−4.13	7
5	III	1.9	−4.10	8
6	II	0.25	−4.04	10

[a] *b/a* is calculated by comparing curve 1 with curves 2–6.

exact than the rest of the curves. This curve has also been omitted in Table I because only one point determines the slope of the steep part of the curve.

Table I is given after the following considerations: The solubility product can be written $DNP^a \times Ca^{++b} = K$. As we have a fairly constant composition of the DNP—the quotient of the OD at 260 and 280 mμ, for instance, being constant—we can write $DNP = k_1$ $DNA = k_2$ OD, where OD is the optical density at 260 mμ and the samples are diluted within the range where Beer's law is valid.

$$k\ OD^a \times Ca^{++b} = K.$$

If two cases, (1) and (2), are considered this gives us at the precipitation point

$$k\,OD_1^a \times C_{Ca_1}^b = k\,OD_2^a \times C_{Ca_2}^b.$$

Introduce the logs and solve for b/a

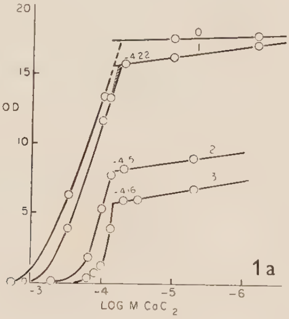
$$b/a = \frac{\log \frac{OD_1}{OD_2}}{\log C_{Ca_2} - \log C_{Ca_1}}$$

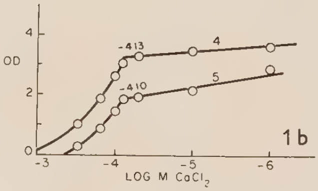
The mean value of b/a in Table I is 7. For the rather limited significance of that figure see discussion below. It should only be pointed out here that it gives some measure of the reproducibility of the experiments. The handling of the samples in the centrifuge tubes directly after centrifugation is certainly the least reproducible point of the method at present. In this connection, it may be noted that curve number 2, which gives the b/a value that deviates most from the mean, was the first curve made, when I had less skill. On the other hand the uncertainty in drawing the curves themselves should be mentioned. This difficulty has for instance been pointed out in curve 1, Fig. 1*a*.

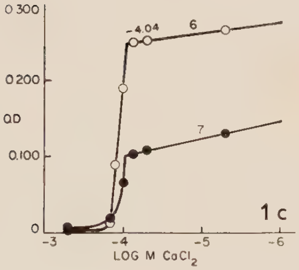
Some of the curves in Fig. 1 have been prepared with different speed of centrifugation of the samples. The rule is that higher speed gives a more inclined line for the part of the curve which has a lower $CaCl_2$ molarity than $10^{-1.2}$, i.e. where DNP is soluble. The lowest OD value of that line is nearest to the precipitation point. A good example is the curves number 0 and number 1. Two parts of each of the samples were centrifuged, one at 2000 × g and the other at 8000 × g for 20 minutes. The OD measurements for the samples treated with the lower speed gave curve number 0, whereas the higher speed gave curve 1.

It may be pointed out that the contamination of NaCl is the same from each pellet within the same series.

In one case (curve 5) the DNP solution in 10^{-6} M $CaCl_2$ was centrifuged in the Spinco analytical centrifuge (UV optics, OD of solution 0.6: 59,870 r.p.m., no buffer). The diagram obtained indicates a fairly large but very heterogeneous material.







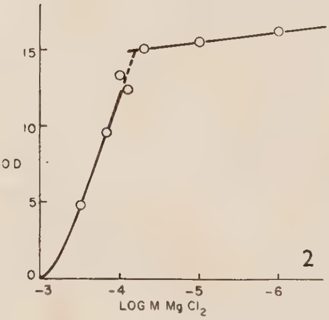


Fig. 1*a*, *b*, *c*.—The solubility of deoxyribonucleoprotein from sea urchin sperms in $CaCl_2$ solutions. The amount of deoxyribonucleoprotein measured by the absorption at 260 $m\mu$ of the supernatant after centrifugation. The samples, giving curves 0 and 6, have been centrifuged at $2000 \times g$ for 20 minutes, all others at $8000 \times g$ for 20 minutes.

Fig. 2.—The solubility of deoxyribonucleoprotein from sea urchin sperms in $MgCl_2$ solutions.

Precipitation with $MgCl_2$.—One set of measurements was made where $MgCl_2$ was substituted for $CaCl_2$. The result, as given in Fig. 2, shows that DNP behaves very much the same in Mg as in Ca solutions. (The $MgCl_2$ solutions were made with the same precautions as mentioned above for $CaCl_2$, the salt coming from a previously unopened bottle of $MgCl_2$, $6H_2O$; Baker code 1909.)

Experiments with deoxyribonuclease.—DNAse (Worthington) was added to a solution of DNP and the sample was kept at 25°C. No drastic difference in the behaviour of this sample towards Ca ions could be found as compared with a sample from the same DNP solution placed at the same temperature but without added enzyme.

When DNP precipitated in 0.17 *M* NaCl as above and shaken with 5×10^{-4} *M* $CaCl_2$ was digested with a 0.0005 per cent DNAse solution, about 30 per cent of the UV-absorbing material went out in the supernatant after 30 hours digestion.

It may also be noted that the viscosity of the DNP solutions dropped when DNAse was added (compare above with sample from tube 1).

Discussion

DNP from sea urchin sperms starts to precipitate at a $CaCl_2$ or $MgCl_2$ concentration of $10^{-4.2}$ *M* (ionic strength 0.0002). As the same amount of the DNP went completely into solution earlier in NaCl of higher ionic strength (0.009) the effect of Ca and Mg must be specific for this latter type of ions.

From the result in Table I the following conclusions can be drawn. The order of magnitude of influence of the Ca ions is very much the same for different preparations of DNP. The quotient of the exponentials of the Ca ion and the DNP concentration in the solubility product will be about 7.

The amount of Ca required to precipitate the DNP (taken to the lower break in the curves) was found to be 0.66, 0.61, and 0.54 per cent of the DNP precipitated for curves 1, 2, and 3, respectively, of Fig. 1*a*. For this calculation the DNP was assumed to contain 35 per cent DNA and the OD of 1 per cent DNA to be 230 at 260 mμ, pH 12. For curves 2 and 3 the maximal amount of DNP in solution was calculated both from OD values using samples without Ca^{++} and from dry weight measurements. For curve 1 the OD only was measured. The dry weight measurements yielded values corresponding to 0.5–0.6 per cent of added Ca per DNP precipitated. Some of the Ca ions added remain, however, in the supernatant. This amount can be calculated from the solubility product for the concentrations at the point in question compared to the product at the precipitation point. In this calculation it is only necessary to use the relative amounts of DNP. For the quotient of the exponents the value 7 is used. The Ca value calculated from curve 1 (Fig. 1*a*) is reduced by $\frac{1}{4}$ for the Ca ions still in the supernatant. For curves 2 and 3 still higher corrections are found. The amount of Ca per DNP precipitated, after correction for the Ca in the supernatant, is about 0.4 per cent. This amount is 8 times as big as that found in the DNP from calf thymus according to Davison and Butler [4]. It should, however, be remembered that the material of Davison and Butler had been earlier brought into solution. At the very point of precipitation the amount of Ca is less. For a solution as strong as the one giving curve 1, i.e 0.23 per cent, the amount of added Ca is 0.10 per cent of the total amount of DNP present. This is only double the amount given by Davison and Butler. To the effect of Ca ions should also be added that of Mg ions. Allgén [1], for instance, reports that Mg is present in all his DNA preparations.

For all practical purposes, where bivalent ions are added to nuclei in suspension or to nuclear material, the strong effect of bivalent ions must be remembered. The aggregation which apparently takes place even at very low Ca ion concentration, as revealed by the sloping of the curve at salt molarities lower than $10^{-4.2}$, must have an influence on many measurements depending on particle size data. The maximum contamination given for NaCl (prepared to A.C.S. standards) is 0.005 per cent Mg and Ca. This means that a solution of physiological saline will contain as much as 10^{-5} *M* of these ions and have the same aggregating effect on the DNP.

The amount of Ca per DNP can also be expressed as nucleotides per Ca. The percentage value given above will correspond to 7–8 nucleotides per added Ca ion, and 11 nucleotides per Ca for the corrected value. The apparent DNP unit as calculated from the solubility product has 7 Ca ions per apparent unit of protein. The order of magnitude of this unit would then be 70,000. It must be kept in mind, however, that the real particles, judging from the ultracentrifuge diagram, differ very much in size as the solution is very heterogenous.

The low effect of the DNAse on the precipitation behaviour of DNP in solutions freed from Mg and Ca ions is not surprising, as the DNA molecule is not very much affected. According to Zamenhof and Chargaff [15] pancreatic nuclease working on DNA in a Mg free medium will give no dialysable fragments at low temperature. The protein moiety may also have some protecting effect on the DNA. The DNA in the DNP precipitated with Ca is partly protected as shown by the enzyme digestion experiment.

The lowering of the viscosity of the DNP solution both from tubes 1 and 2 by the DNAse is of interest, as it shows that there are nucleic acid groups free for the enzyme to attack. The Ca ions added are much more likely to stick to the phosphate group of the DNA than to precipitate with any of the carbonyl groups of the protein. That there should be specifically oriented protein groups with high affinity for Ca is unlikely. Semenza [14] has shown that the DNA from calf thymus sticks much harder to the Ca in a Ca phosphate column than the protein does. The tobacco mosaic virus can be used as a model complex of nucleic acid (in this case RNA) and protein. Its behaviour on a Ca phosphate column was studied by the author. If the virus is heated at pH 8.3–8.5 to 35°C for 15 minutes most of the virus will be eluted as the nucleic acid fraction. The same treatment at a lower pH will

Leave the virus more intact and it will all come out as protein, whereas a rise of pH to 8.8–9.2 and of temperature to 50–55° will split the virus. Protein and nucleic acid will leave the column separately eluted in their appropriate places. I am tempted to look upon the first case as an obvious example of the over-all influence of a few liberated phosphate groups that stick out, and attach the rest of particle to the column. The changes in the charge effect of the phosphate group of some of the DNA in the DNP would suffice to explain the sensitivity of the DNP in sea urchin sperms for added Ca or Mg ions. More experimental data, however, are necessary to judge on the validity of the hypothesis of Mazia, especially in regard to the criticism of Kaufmann and McDonald. The obvious experiments are, for instance, to test the effect of chelating agents of different capacities.

SUMMARY

Deoxyribonucleoprotein (DNP) prepared from sea urchin sperms with the use of citrate and 0.009 *M* NaCl solutions is precipitated by very small amounts of Ca or Mg ions. The precipitation starts for a 0.2–0.1 per cent DNP solution at $10^{-4.2}$ *M* $CaCl_2$ or $MgCl_2$. At lower salt concentrations the Ca ions have an aggregating effect on the DNP molecules. Precipitated DNP (with 35 per cent DNA) contains about one Ca ion per 11 nucleotides. In a $CaCl_2$-DNP concentration diagram the change in the precipitation point seems to fulfil the requirements of an ordinary solubility product with about 7 Ca ions per apparent unit of DNP. The DNP precipitated with Ca ions can be brought back into solution by treatment with citrate.

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UNTERSUCHUNGEN ZUM NACHWEIS DER ALKALISCHEN PHOSPHATASE AN IN VITRO GEZÜCHTETEN MENSCHLICHEN CARCINOMZELLEN MIT BESONDERER BERÜCKSICHTIGUNG IHRER BEZIEHUNG ZU DER ZELLOBERFLÄCHE[1]

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Eingegangen am 25. November, 1957

Im Laufe von Untersuchungen über die intrazelluläre Lokalisation von histochemisch nachweisbaren Fermenten an in vitro gezüchteten Geweben [9] und im Besonderen an menschlichen Carcinomzellen des HeLa-Zellstamms hat sich ergeben, dass an diesen Zellen die alkalische Phosphatase eine besondere Beziehung zu der Zelloberfläche bzw. den Zellgrenzflächen aufweist. Dieser Befund musste umsomehr Interesse beanspruchen, da der Zelloberfläche, wenigstens für die meisten Warmblüterzellen, der Charakter eines eigenen, von dem Plastosomen verschiedenen, ektoplasmatischen Strukturelement häufig abgestritten wird (s. auch [18]). Selbst die Elektronenmikroskopie konnte bisher nur durch mittelbare Rückschlüsse zu wohl einleuchtenden, aber doch eben hypothetischen Vorstellungen über die Struktur der Zelloberfläche bzw. des Plasmalemma kommen (zusammenfassende Darstellung bei Frey-Wyssling [5]). Im Ganzen sind es mehr physiologische Befunde über den Stoffaustausch (s. Übersicht bei Rosenberg und Wilbrandt [16]) oder über die Beeinflussbarkeit der Zelloberflächenbewegungen [11], die die Existenz eines höher strukturierten Baues einer Zellmembran annehmen lassen. Dabei bietet die morphologische Beobachtung lebender Zellen im Phasenkontrastmikroskop an der Zelloberfläche eine Fülle von Erscheinungen: Bewegungen und Faltungen mannigfacher Art, Ausbildung und Rückbildung von Fortsätzen, Phagocytose, Pinocytose u. a. m., freilich ohne mehr als eine einfach konturierte, dünne Zellgrenze erkennen zu lassen.

In der vorliegenden Untersuchung soll in erster Linie die Lokalisation der alkalischen Phosphatase an Zellgrenzflächen von HeLa-Carcinom-Zellen demonstriert und durch Versuche mit Einwirkung von Stoffen, die an der Zelloberfläche angreifen, sichergestellt werden.

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[1] Der Deutschen Forschungsgemeinschaft und der Gesellschaft zur Bekämpfung der Krebskrankheiten, Nordrhein-Westfalen, e.V., danken wir für ihre Unterstützung.

MATERIAL UND METHODE

Bei dem HeLa-Carcinomzellstamm handelt es sich um ein seit 6 Jahren, zuerst von Gey [7], in vitro gezüchtetes, menschliches Epidermoidcarcinom der Cervix uteri. Für die vorliegende Untersuchung wurde es in Form von Suspensionskulturen in 150 bzw. 180 ml fassenden Kulturflaschen nach Kolle oder nach Breed–Demeter verwendet. Dabei wird der Boden dieser Flaschen mit Deckglasstreifen belegt, eine Suspension von HeLa-Zellen einpipettiert und mit Nährflüssigkeit soweit aufgefüllt, dass der Boden ausreichend bedeckt ist. Schon nach kurzer Zeit setzen sich die Zellen auf der Glasoberfläche ab. Innerhalb weniger Tage vermehren sie sich soweit, dass man neben einzeln liegenden Zellen kleinere Zellkomplexe bis ausgedehnte, zusammenhängende membranartige Verbände in einschichtiger Lage von sehr flacher Ausbreitung findet. Die Nährflüssigkeit besteht aus einer 0,5%igen Lösung von Lactalbuminhydrolysat (NBC, Cleveland, Ohio) in Hanks-Lösung + 5 % Plazentarserum.

In einigen Fällen wurden gleichartige Kulturen eines menschlichen Epidermoidcarcinoms des Larynx, Stamm A [4] verwendet. Das Züchtungsmedium musste hierfür durch zusätzliches Plazentarserum und Embryonalextrakt ergänzt werden.

Die ausreichend bewachsenen Deckglasstreifen wurden zur Durchführung von Fermentreaktionen und Kontrollfärbungen, darunter die PAS-Reaktion, unmittelbar in 2%igem, neutralen Formalin bei 4°C für mindestens 2 und nicht länger als 4 Stunden fixiert. In mehreren Versuchen wurden die Zellen innerhalb der Kulturflaschen einer *Vorbehandlung* mit Substanzen, die das Verhalten der Zelloberfläche beeinflussen, wie Versene oder Trypsin, in einigen Fällen auch Adenosintriphosphorsäure (ATP, Boehringer), unterworfen (s.u.) und dann in gleicher Weise fixiert.

Fermentreaktionen: Alkalische Phosphatase, pH 9,2.

(*a*) Nach der Takamatsu Gomori-Methode [8], Substrat: Na-β-glycerophosphat, Inkubationszeiten 15–75 Min. bei 37°C; Kerngegenfärbung mit Mayers Hämalaun.

(*b*) Nach der Azo-Kupplungsmethode (Pearse) [13], Substrat: Na-α-naphthylphosphat; Inkubationszeit 10–15 Min. bei Zimmertemperatur; keine Gegenfärbung.

(*c*) In einigen Ansätzen wurde an unfixierten Kulturen die Azokupplungsmethode durchgeführt; nach Inkubationszeiten von 7–10 Min. und kurzem Spülen in physiologischer Salzlösung Fixierung in 2%igem neutralen Formalin [9].

In allen Versuchen wurden als Kontrollen Kulturen (*a*) ohne Substratzusatz und *b*) nach Hitzezerstörung des Ferments in substrathaltiger Lösung inkubiert. Sie blieben durchweg negativ.

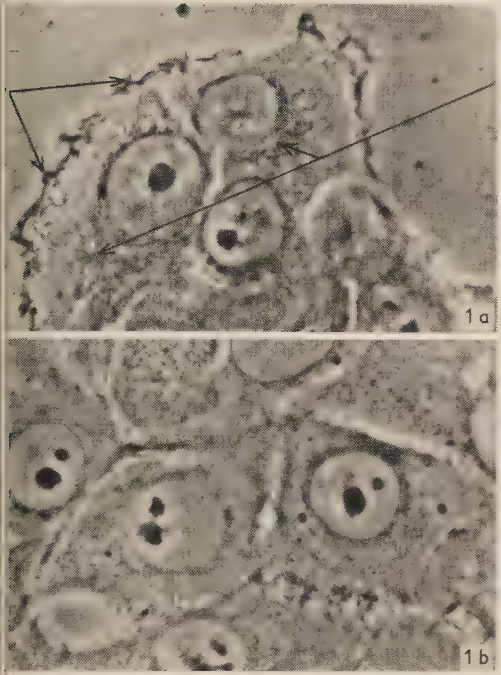
Die Untersuchung an fixierten Zellen wurden durch Beobachtungen lebender HeLa-Carcinomzellen im Phasenkontrastmikroskop, insbesondere auch unter der Einwirkung von Versene, Trypsin und ATP ergänzt. Auch die Präparate, an denen Fermentreaktionen durchgeführt waren, wurden zur besseren Lokalisierung des Reaktionsproduktes regelmässig im Phasenkontrastmikroskop kontrolliert.

ERGEBNISSE

An lebenden, flach ausgebreiteten HeLa-Carcinomzellen — und den in ihrem Aussehen und Verhalten sehr ähnlichen Zellen des A-Stammes — zeigt die Beobachtung im Phasenkontrastmikroskop lebhafte Bewegungen und Formveränderungen der Zelloberfläche. An den freien Zellrändern fallen ganz besonders Faltungen (Abb. 1*a*) auf, die sich ständig verändern und wie Wellen über die Zelloberfläche hinwegwandern, ein stetiges Wogen, das in phasenkontrastkinematographischen Serien [6, 12] sehr anschaulich gemacht werden kann. Eng verknüpft mit diesem Bewegungstypus der Oberfläche ist das Phänomen der Pinocytose [6]. Andere Zellen sind an ihren freien Rändern bis zur Grenze der Sichtbarkeit dünn ausgebreitet, entweder mit einem glatten Saum oder mit zahlreichen feinst ausgezogenen Fortsätzen. In jedem Fall sind diese Formen der Oberfläche nicht starr, sondern wandeln sich ständig und gehen ineinander über. Dies gilt auch für die Zellgrenzen der innerhalb von einschichtigen, membranartigen Verbänden benachbarten Zellen. Ein Maschenwerk zarter, fadenförmiger Oberflächenfortsätze (Abb. 1*b*), von sehr viel feinerer Ausbildung als die bekannten Interzellularbrücken des normalen Plattenepithels, verzahnt benachbarte Zellen und besitzt sicher eine besondere Bedeutung für den gegenseitigen Zusammenhalt der Zellen. Ähnlich feine Oberflächenfortsätze dürften das Haften der Zellen an der Glasoberfläche gewährleisten [2]. Eine ausserzelluläre Substanz im Sinne eines interzellulären Zements ist bei der phasenkontrastmikroskopischen Beobachtung unserer Kulturen nicht erkennbar.

Nachweis der alkalischen Phosphatase an HeLa-Zellkulturen

Mit der *Calcium-Cobalt-Methode* [8] stellen sich bei allen verwendeten Inkubationszeiten von 15–75 Min. mit grosser Regelmässigkeit und besonders scharfer Zeichnung die Zellgrenzen dar. Innerhalb von membranartigen Verbänden liegende Zellen zeigen in ihrer gesamten Circumferenz eine positive Fermentreaktion der verzähnelten, benachbarten Zellränder (Abb. 2*a*). Häufig kann man die Doppelkontur der gegenüberliegenden Zellgrenzen mit einem feinen Spalt erkennen. Anders verhält es sich mit den freien Säumen von einzeln oder am Rand von Verbänden liegenden bzw. an Lücken angrenzenden (Abb. 2*b*) Zellen: Sie sind sehr häufig fermentnegativ, insbesondere dort, wo die Zelle einen dünn ausgebreiteten Randsaum hat. Nur an Stellen, an denen vielfache, feine, fadenförmige Fortsätze des Zellrandes vorhanden sind, tritt eine deutliche positive Fermentreaktion auf (Abb. 2*b*). Dagegen stellen sich Falten der Zelloberfläche, wie sie die Abb. 1*a* an der



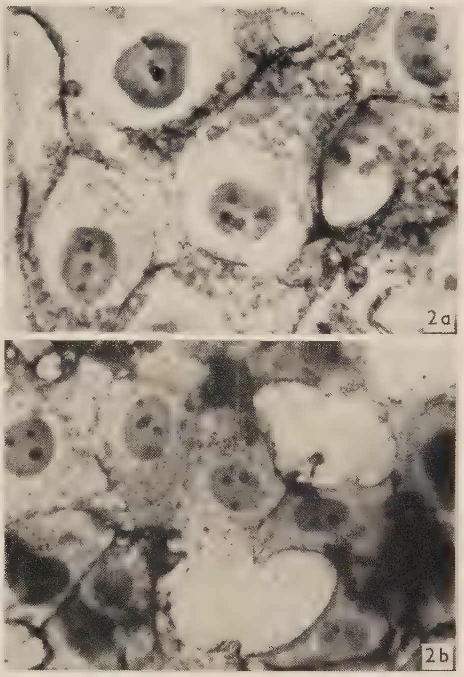


Abb. 1.—HeLa, lebend, Phasenkontrast; Vergr. 640 ×. *a*, Falten der Zelloberfläche→; Mitochondrien←. *b*, Zellverband; Verzahnung benachbarter Zellgrenzen durch feinste Oberflächenfortsätze.

Abb. 2.—HeLa, alkalische Phosphatase (Gomori); Gegenfärbung Mayers Hämalaun. *a*, Zeichnung der Zellgrenzen durch das Reaktionsprodukt; Inkubation 37 Min.; Vergr. 800 ×. *b*, Fadenförmige Fortsätze der Zellränder weisen gleichfalls Fermentaktivität auf; Inkubation 75 Min.; Vergr. 560 ×.

lebenden Zelle zeigt — sie bleiben bei der Fixierung recht gut erhalten — nicht klar. Eine sehr starke Fermentreaktion zeigt die Oberfläche von Zellen während der Mitose, insbesondere auch an den zahlreichen Ausziehungen der Zelloberfläche (Abb. 3), wie sie als Retraktionsspuren bei der Abrundung der Zellen entstehen und in dieser Weise mit andern färberischen Methoden kaum sichtbar gemacht werden können.

Innerhalb des Cytoplasmas der HeLa-Zellen lässt sich mit der Calcium-Kobaltmethode, häufig erst bei höheren Inkubationszeiten von 40–70 Min., ein meist granuläres, gelegentlich feinfädiges bis stäbchenförmiges Reaktionsprodukt nachweisen. Die Reaktion im Cytoplasma ist von Zelle zu Zelle etwas verschieden und tritt in ihrer Stärke deutlich hinter der Lokalisation an den Zellgrenzen zurück. Zu diffusen Anfärbungen im Cytoplasma kommt es nicht. Das Reaktionsprodukt des Fermentnachweises ist immer scharf gezeichnet. Der Zellkern und die Mitosechromosomen sind stets frei von

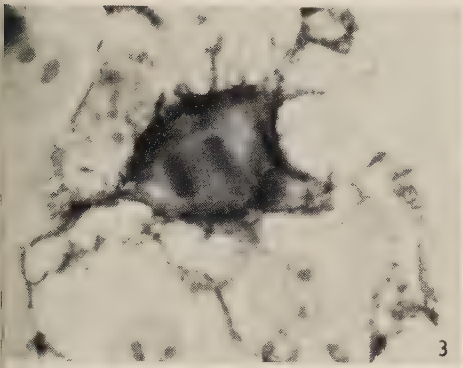
Fermentaktivität. Allein der Nucleolus weist gelegentlich eine ganz zarte, diffuse Bräunung auf, die als unspezifisch angesehen werden kann. Substratansätze ohne Zugabe von Mg-Ionen hatten keine Abschwächung der Fermentreaktion zur Folge.

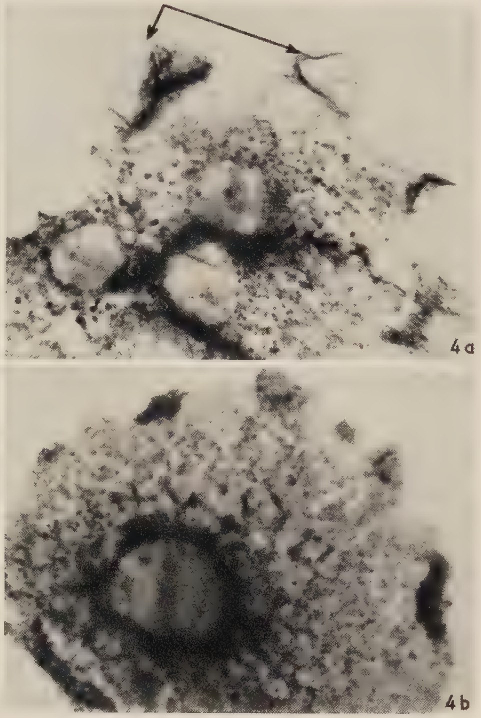
Das Ergebnis der *Azokupplungsmethode* zum Nachweis der alkalischen Phosphatase an *fixierten* HeLa-Zellen unterscheidet sich von dem der Calcium-Cobalt-Methode in einigen Punkten: die Zellen — einzeln, wie in Verbänden liegende — sind durchweg stark positiv; das fein- bis gröber-granuläre, oft auch feinfädig abgelagerte Reaktionsprodukt ist mit Ausnahme des Kernraums über den ganzen Zelleib verteilt (Abb. 4*a* and *b*). Eine Zeichnung der Zellgrenzen, wie sie oben bei der Calcium-Cobalt-Methode beschrieben wurde, fehlt meist. Bei der reichlichen Ablagerung des Reaktionsproduktes ist es manchmal schwierig zu entscheiden, ob dieses innerhalb des Cytoplasmas oder an der Zelloberfläche liegt. Sehr häufig werden in den dünn ausgebreiteten, peripheren Zellbezirken die Zelloberfläche und gröbere Oberflächenfalten freier Zellränder (Abb. 4*a*) durch das Reaktionsprodukt deutlich dargestellt.

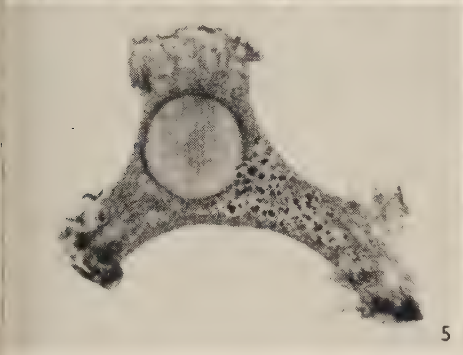
Versuche, die Azokupplungsmethode an *unfixierten*, lebenden HeLa-Zellen durchzuführen, wurden aufgrund der Vorstellung unternommen, dass bei kurzen Inkubationszeiten das Substrat und das zur Kupplung verwendete Diazoniumsalz nicht so rasch in die Zellen eindringen könnten. Dadurch müsste eine positive Reaktion der Zelloberfläche deutlicher werden. Nach Inkubationszeiten von 7–10 Min. wurden die Zellkulturen in Formalin fixiert. Tatsächlich wird an den HeLa-Zellen bei diesem Vorgehen die Zelloberfläche mit ihren feinsten Faltungen an den Zellrändern durch das Reaktionsprodukt dargestellt (Abb. 5). Die gewonnenen Bilder decken sich weitgehend mit dem Bild der lebenden Zelle im Phasenkontrastmikroskop. Ausserdem kommt es im Cytoplasma zu einer ähnlichen, wenn auch oft schwächeren, granulären Verteilung des Reaktionsproduktes wie an vorher fixierten Kulturen. In dem zwar isotonischen, aber stark alkalischen Milieu (pH 9,2) der Inkubationslösung treten keine gröberen Veränderungen am Aussehen der Zellen auf.

Die Zellen des zum Vergleich mit den HeLa-Zellen untersuchten menschlichen Epidermoidcarcinoms, Stamm A, sind bei den angewandten Methoden zum Nachweis der alkalischen Phosphatase fast völlig negativ.

Die PAS-Reaktion an Formol-fixierten HeLa-Zellkulturen ergibt ein Bild, das dem Ergebnis der Calcium-Cobalt-Methode ähnlich ist. Man findet zwar eine schwache, aber deutlich positive Reaktion an den Grenzen benachbarter Zellen und eine granuläre Anfärbung im Cytoplasma.







bb. 3. -HeLa; alkalische Phosphatase (Gomori); Inkubation 75 Min.; Gegenfärbung mit Mayers ämalaun: Anaphase, mit fädigen Retraktionsspuren des Cytoplasmasaums. Vergr. 790 ×.

bb. 4.—HeLa, alkalische Phosphatase (Azokupplungsmethode); Inkubationszeit 10 Min.; eine Gegenfärbung. *a* and *b*, granuläres Reaktionsprodukt vorwiegend im Cytoplasma. In *a*, utliche Darstellung von Zelloberflächenfalten →. Die fermentnegativen Kernräume teilweise in nscharfer Einstellung. *a*, Vergr. 736 ×, *b*, Vergr. 840 ×.

bb. 5.—HeLa, alkalische Phosphatase (Azokupplungsmethode) an *unfixierter* Zelle; Inkubations-it 7 Min., anschl. Formolfixierung; keine Gegenfärbung. Aktivität im Cytoplasma und an den berflächenfalten der Zellränder. Vergr. 735 ×.

orbehandlung der Zellkulturen

In mehreren Versuchen wurden HeLa-Zellkulturen mit 0,05%igem Tryp- n (DIFCO) oder 0,02%igem Versene (Dinatriumsalz der Äthylendiaminte- aessigsäure), jeweils bei pH 7,2–7,3, in $Ca^{\cdot\cdot}$-und $Mg^{\cdot\cdot}$- freier physiologischer alzlösung vorbehandelt. Beide Substanzen verursachen innerhalb gewisser inwirkungszeiten (10–30 Min. bei Trypsin; 30–45 Min. bei Versene) eine uflösung der membranartigen Zellverbände und eine Ablösung der Zellen on der Glasunterlage. Diese Ablösung kann mechanisch durch Schütteln der leichtes Abspülen der Glasfläche wesentlich beschleunigt werden.

Trypsin und Versene werden deshalb in den gleichen Konzentrationen be der laufenden Weiterzüchtung der HeLa-Zellkulturen zur Herstellung von Suspensionen isolierter Zellen benutzt. Dabei kommt es innerhalb der genannten Einwirkungszeiten nicht zu einer nachweisbaren Zellschädigung. Quantitative Beobachtungen ergaben, dass sich die so behandelten Zellen nach Aufschwemmung in neuem Nährgemisch innerhalb kurzer Zeit vollzählig wieder auf der Glasunterlage des Züchtungsgefässes ausbreiten und vermehren.

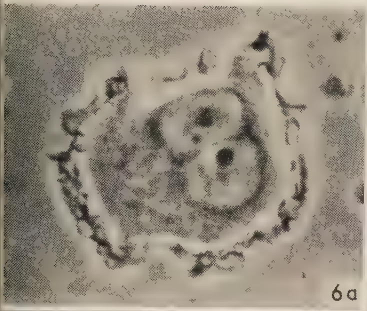
Wie Beobachtungen an lebenden HeLa-Zellen im Phasenkontrastmikroskop zeigen, bewirken Trypsin und Versene zuerst eine Auflockerung der Zellverbände. Die Zellen retrahieren die flachen, peripheren Cytoplasmasäume und runden sich zunehmend ab. Dabei bleiben zwischen den Zellen und an der Glasunterlage zahlreiche, von der Zelloberfläche ausstrahlende, feinste, langausgezogene Fäden als „Haftfäden" bestehen. Bei länger dauernder Einwirkung des Versene verschwinden manchmal auch diese und die mehr oder minder abgerundeten Zellen haben grossenteils eine glatte Oberfläche. Dagegen bleiben beim Trypsin die „Haftfäden" verhältnismässig lange erhalten. Trypsin, weniger häufig Versene, ruft jedoch ausserdem an vielen Zellen — in der Hauptsache an den einzeln oder am Rande von membranartigen Verbänden liegenden — eine eigenartige Verstärkung der Bewegungen der Oberflächenfalten hervor. Es kommt in diesem Fall nicht zu einer Retraktion und Abrundung der Zelle, sondern nur zu einer Dickenzunahme im Zentrum um den Kern. Die peripheren Cytoplasmasäume bleiben ausgebreitet. Die Oberflächenfalten an diesen Säumen sind vermehrt und vergröbert (Abb. 6*a* und 6*b*), und ihre Bewegung ist stark beschleunigt. Man beobachtet dabei, wie sie ständig dünne, cytoplasmatische Säume ausbilden und dann die Oberflächenfalten von innen nach aussen wellenförmig über sie hinwegwandern.

Wenn die Glasunterlage nach etwa 5-10 Min. (Trypsin) bzw. 15–30 Min.

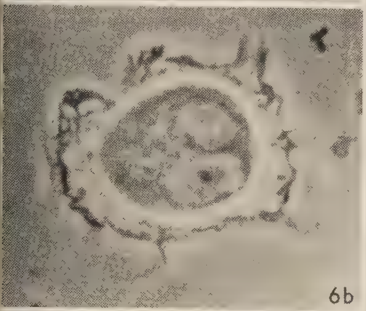
Abb. 6.—HeLa-Zelle, lebend; Phasenkontrast. Einwirkung von Trypsin: *a*, nach 4 Min.; *b* nach 9 Min., Dickenzunahme im Zentrum der Zelle, verstärkte Oberflächenfalten in der Peripherie; *c*, am Glas zurückgebliebene Haftspur derselben Zelle kurz nach leichtem Abspülen (24 Min. nach Versuchsbeginn). Vergr. 720 ×.

Abb. 7.—HeLa-Zellen, nach 40 Min. Versene-Einwirkung; alkalische Phosphatase (Gomori); Inkubation 75 Min.; Gegenfärbung mit Mayers Hämalaun. Fermentaktivität nur noch an den Behrührungsstellen der Zellen des sich auflösenden Verbandes. Vergr. 720 ×.

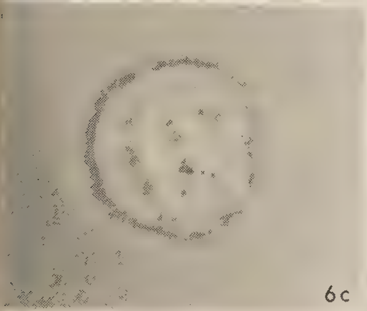
Abb. 8.—HeLa, nach 5 Min. Trypsin-Einwirkung; alkalische Phosphatase (Azokupplungsmethode); Inkubation 10 Min.; keine Gegenfärbung. — Am Glas haftende Reste der Zelloberfläche neben noch nicht abgelösten Zellen. *a*, Vergr. 514 ×; *b*, Vergr. 902 ×.



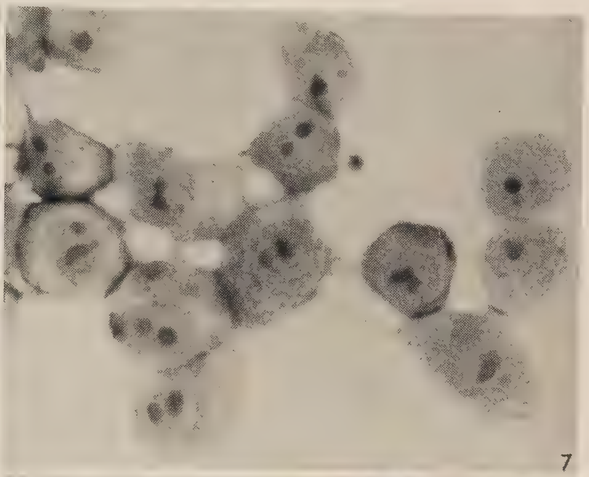
6a



6b



6c



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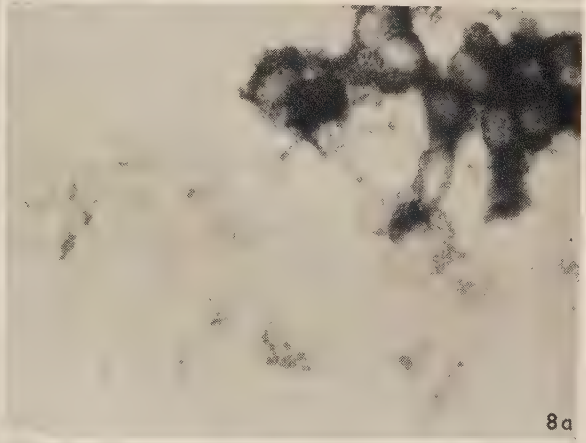
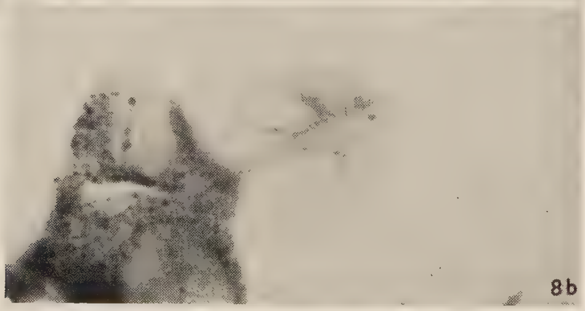


8a



8b

(Versene) leicht mit physiologischer Salzlösung abgespült und damit ein Grossteil der Zellen abgelöst wird, dann bleiben an der Glasunterlage Reste der Zelloberflächen haften (Abb. 6*c*). Diese bewahren ähnlich einem Abdruck die Konturen der abgelösten Zellen oft bis in feine Details der Zellformen. Meist bleiben einige Granula in Zusammenhang mit diesen Resten der Zelloberfläche. Wenn die mechanische Ablösung der Zellen durch Abspülen nach länger dauernder Einwirkung des Trypsin (bis 30 Min.) vorgenommen wird, so haben die an der Glasunterlage haftenden Zellreste häufig die Form glattwandiger Bläschen. Sie enthalten gewöhnlich einige Granula in Brownscher Bewegung und ähneln den Bläschen, wie sie von der Potocytose [18] her bekannt sind.

Völlig gleichartige phasenkontrastmikroskopische Beobachtungen konnten an Kulturen des A-Carcinomzellstamms gemacht werden. Diese Zellen lösen sich allerdings viel leichter und in kürzerer Zeit als die HeLa-Zellen von der Glasunterlage ab.

Mit ATP wird zunächst eine ähnliche Wirkung wie mit Trypsin und Versene erzeugt, nämlich eine Retraktion und Dickenzunahme der Zellen unter Ausbildung von fädigen Haftfortsätzen der Zellen untereinander bzw. an der Glasunterlage. Die Zellen lösen sich dann jedoch nicht ab. Bei stärkeren Dosen (5 mg/ml und Einwirkung bis 4 Stunden) kann man an vielen Zellen die Entstehung kleiner Bläschen ("blisters") an der Zelloberfläche beobachten.

Nachweis der alkalischen Phosphatase an vorbehandelten Zellkulturen

An HeLa-Zellkulturen, die in der beschriebenen Weise vorbehandelt waren, wurde nach Formalinfixierung die Calcium-Cobalt- und die Azokupplungsmethode zum Nachweis der alkalischen Phosphatase durchgeführt. Bei Wahl des richtigen Zeitpunktes nach Trypsin- oder Versene-Einwirkung, der sehr wechseln kann, wird beim Eintauchen des bewachsenen Glasstreifens in die Fixationsflüssigkeit ein Teil der Zellen abgespült, während ein anderer auf der Glasfläche haften bleibt. Nach der Fixierung lösen sich keine Zellen mehr von der Glasunterlage ab.

Die Haftfäden der Zelloberfläche, die bei der durch Versene oder Trypsin verursachten Auflösung der Zellverbände und der Abrundung der Zellen entstehen, sind mit beiden Nachweismethoden der alkalischen Phosphatase immer stark fermentpositiv. Kulturen, die mit Versene behandelt waren, zeigen ausserdem bei der Calcium-Cobalt-Methode eine deutliche Abnahme der Fermentreaktion an den Grenzen benachbarter Zellen, die mit der fortschreitenden Auflockerung und Auflösung der Zellverbände Hand in Hand eht (vergl. Abb. 7 mit Abb. 2*a* und *b*). Die Abbildung 7 zeigt die Zellen mehr der minder abgerundet. Die Grenzen, an denen sie sich berühren, sind schmaer geworden und *nur* noch dort findet sich eine Fermentaktivität. Wo das ersene noch nicht genügend stark gewirkt hat und noch membranartige ellverbände bestehen, sind die Verhältnisse noch genau so, wie es die Abbilung 2 darstellt.

Die an der Glasunterlage haftenden Reste von Zellen, die infolge der Trypin- oder Versene-Vorbehandlung bei der Fixierung mechanisch abgelöst orden sind, geben mit beiden Nachweismethoden der alkalischen Phoshatase eine etwa gleichartige, positive Reaktion (Abb. 8*a* und *b*). Unpezifische Niederschläge oder die Abwanderung des Reaktionsprodukts aus ellen, die sich erst nachträglich abgelöst haben könnten, sind mit Sicherheit ufgrund entsprechender Kontrolluntersuchungen auszuschliessen. Unter anerem wurde das Ferment an den am Glas verbliebenen Teilen der Zelloberäche in Präparaten nachgewiesen, in denen sich nach phasenkontrastnikroskopischer Kontrolle keine intakten Zellen mehr befanden. An Trypsin- der Versene-vorbehandelten Stamm-A-Zellkulturen blieben entsprechend em Fehlen nachweisbarer alkalischer Phosphatase auch die im Phasenkonastmikroskop beobachteten Haftspuren der Zelloberfläche am Glas negativ.

Die alkalische Phosphatase ist an Zellen, die mit ATP behandelt waren, n stärkerem Masse als es sonst gefunden wurde positiv. Da die feinen, on der Oberfläche der retrahierten Zellen ausstrahlenden Haftfäden sehr ermentaktiv sind, ist in diesem Falle häufig die gesamte Zelle, mit Ausahme des fermentnegativen Kerns, intensiv geschwärzt, ohne dass Einzeleiten erkannt werden können. Bläschen der Zelloberfläche (“blisters”), die nter der ATP-Wirkung entstehen, sind mit einem feinen, fermentpositiven berzug versehen.

BESPRECHUNG

Wenn hier der Versuch gemacht wurde, ein histochemisch nachweisbares erment innerhalb der Zelle zu lokalisieren, so muss darauf hingewiesen erden, dass die verwendeten Zellkulturen dafür besonders geeignet sind. ie in einschichtiger Lage flach ausgebreiteten Zellen erlauben sehr kurze ixierungs- und Inkubationszeiten. Dadurch werden Fermentverluste und iffusionserscheinungen weitgehend eingeschränkt. Sie können, wie auch ntersuchungen zum Nachweis weiterer Fermente, u. a. von Esterasen, zeigen], bei Verwendung unfixierter Zellkulturen noch weiter verringert werden. ies mag zum Teil darauf beruhen, dass dabei die Inkubationszeiten erhebch kürzer gewählt werden können.

Mit beiden Nachweismethoden der alkalischen Phosphatase ist an HeLa-Zellen eine starke Fermentaktivität beobachtet worden, ganz im Gegensatz zu den morphologisch sehr ähnlichen Zellen des A-Stamms, die sich fast völlig negativ verhalten. Beim Vergleich der mit beiden Methoden erzielten Ergebnisse an den HeLa-Zellen finden sich einige Unterschiede. Mit der weniger empfindlichen Calcium-Cobalt-Methode (Gomori) wird fast elektiv das Ferment an den Grenzen benachbarter Zellen dargestellt. Bei der Azokupplungsmethode fehlt eine besondere Aktivität an den Grenzen benachbarter Zellen. Bei dieser Methode ist jedoch immer eine Fermentaktivität im Cytoplasma vorhanden. Aber auch die Oberflächenfalten der freien Randsäume der Zellen sind fermentpositiv, wie dies besonders deutlich das Ergebnis der Azokupplungsmethode an unfixierten Zellen zeigt, während sie mit der Calcium-Cobaltmethode meist nicht zur Darstellung kommen. Die nach Ablösung der Zellen durch Trypsin oder Versene an der Glasunterfläche haftenden Reste der Zelloberfläche, die auch einige Granula aus dem oberflächennahen Cytoplasma enthalten, besitzen an HeLa-Zellen mit beiden Nachweismethoden eine gleich starke und gleichartig lokalisierte Fermentaktivität; bei den Stamm-A-Zellen dagegen sind sie negativ.

Es besteht kein Anhalt dafür, dass der positive Ausfall der Calcium-Cobaltreaktion an den Zellgrenzen auf einem Diffusionsartefakt beruht. Die Versuche zum Nachweis des Ferments nach Versene-Vorbehandlung wurden zum Teil eigens zur Ausschaltung dieser Möglichkeit angestellt. Sie haben an HeLa-Zellen gezeigt, dass nach einer elektiven Einwirkung auf die Zelloberfläche eine echte Beziehung zwischen der Lokalisation der Fermentaktivität bei der Calcium-Cobaltmethode und den Zellgrenzen deswegen besteht, weil das Ferment nur dort nachweisbar bleibt, wo Zellen noch in unmittelbarem Kontakt aneinander grenzen (vgl. Abb. 7).[1]

Das teilweise unterschiedliche Ergebnis der Fermentlokalisation an HeLa-Zellen bei beiden Fermentnachweismethoden führt zu der Frage, ob verschiedene, substratspezifische Fermente nachgewiesen werden oder ob ein und dasselbe Ferment in den einzelnen Bezirken der Zelle eine unterschiedliche Sensibilität gegenüber den beiden angewandten Methoden aufweist. Da bei der Calcium-Cobaltmethode β-Glycerophosphat, bei der Azoupplungsmethode α-Naphthylphosphat als Substrat verwendet wird, betünde die Möglichkeit, dass beide Methoden innerhalb der gleichen Zellen wei verwandte Phosphatasen von gleichem pH-Optimum mit verschiedener pezifität für beide Substrate zur Darstellung bringen. Sichere und unwiderprochene Hinweise für diese Annahme aufgrund biochemischer Unteruchungen fehlen nach unserer Kenntnis (s. a. [15]). Andererseits lässt sich nnehmen, dass die an den einzelnen Orten der Zelle jeweils besondere trukturbindung einer einheitlichen alkalischen Phosphatase ein unterchiedliches Verhalten gegenüber verschiedenen Substraten bedingen [14] nd die Ursache für das auseinanderweichende Ergebnis bei beiden achweismethoden sein kann. Diese Fragen können jedoch mit Hilfe der isherigen Untersuchungen nicht entschieden werden.

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[1] Da Versene auch Mg-Ionen bindet und andererseits bekannt ist, dass die alkalische Phosphatase durch Mg-Ionen aktiviert wird [10], bestände die Möglichkeit, dass bei der vitalen Einwirkung des Versene die alkalische Phosphatase einfach nur gehemmt wird. Diese Hemmung würde bei der Durchführung der Calcium-Cobaltmethode wieder rückgängig gemacht, da dem Substratansatz in der Regel $MgSO_4$ zugefügt wird. Aber auch nach Auslassung des $Mg^{\cdot\cdot}$ aus dem Substratansatz an Versene vorbehandelten Zellen war das Reaktionsergebnis unverändert. Ein aus der gleichen Überlegung heraus geprüfter Substratansatz für die Azokupplungsmethode, dem 0,1 % Versene zugesetzt war, liess keine nachweisbare Hemmung der Fermentreaktion erkennen.

In elektronenmikroskopischen Untersuchungen haben Borysko und Sapraauskas [2] gezeigt, dass auf einer Glasunterlage wachsende Zellen an ihrer laszugewandten Oberfläche zahlreiche feine Fortsätze besitzen. Offenbar aften sie mit diesen so fest an der Glasunterlage, dass ein Teil der Zelloberäche am Glas verbleibt, wenn sich die Zellen infolge der Trypsin- oder Verenewirkung ablösen. Versene und Trypsin haben bei ähnlicher Wirkung erschiedene Angriffspunkte an der Zelloberfläche: Versene, ein Komplexildner, der insbesondere $Ca^{\cdot\cdot}$ und $Mg^{\cdot\cdot}$ bindet, entfernt das für den Zellusammenhalt wichtige Calcium [1, 3]; das proteolytische Trypsin dürfte n seiner Einwirkung auf die lebende Zelle auf Proteine der gleichen äusserten Schicht der Zelloberfläche beschränkt bleiben, die auch das Calcium nthält. Vermutlich sind es Teile dieser Schicht, die beim Ablösen der Zellen m Glas haften bleiben und alkalische Phosphatase enthalten. Das mit der alcium-Cobaltmethode an den Zellgrenzen und das mit der Azokupplungsethode an den Zelloberflächenfalten gefundene Ferment liegt in dieser usseren, von Trypsin und Versene angreifbaren Schicht der Zelloberfläche. hre Beeinflussung durch die beiden Stoffe zeigt, dass diese äussere Schicht er Zelloberfläche vor allem für den gegenseitigen Zusammenhalt („mutual dhesiveness“ [3]) der Zellen und ihr Haften an der Glasunterlage verantwortch ist. Welche Bedeutung das Vorhandensein der alkalischen Phosphatase n dieser Lokalisation bei HeLa-Zellen hat, ist unbekannt. Es ist auffallend, ass die gleichen Bezirke der Zelloberfläche der Stamm-A-Zellen, die sich nter der Trypsin- und Versenebehandlung ähnlich wie HeLa-Zellen veralten, fermentnegativ sind.

Sofern Befunde über die Lokalisation von Fermenten an Zellgrenzen voriegen (s. [16]), haben sie unserer Meinung nach keine Beziehung zu der n der Oberfläche von HeLa-Zellen nachgewiesenen alkalischen Phosphatase.

Die häufig wiederholte Beobachtung der Lokalisation von alkalischer Phosphatase an den Randsäumen von Dünndarmepithelien und den Bürstensäumen von Tubulusepithelien der Niere ist bei der ganz eigenen Bedeutung dieser Strukturen für die Stoffaufnahme aus den Lumina, die sie begrenzen, nicht ohne weiteres mit dem hier geschilderten Vorkommen dieses Fermentes zu vergleichen. Phosphatasen, die von Rothstein an der Oberfläche von Hefezellen nachgewiesen wurden [17], stehen offenbar im Dienste des Glucosetransportes.

Es stellt sich die Frage, ob die Schicht der Oberfläche der HeLa-Zellen, in der die alkalische Phosphatase gefunden wurde, ein Bestandteil der Zelloberfläche (im Sinne einer gelartigen Plasmamembran [6]) ist oder einer äusseren „Bindeschicht", d.h. einer extrazellulären Zementsubstanz, zugehört. Obwohl diese Frage aufgrund dieser Untersuchungen nicht ganz entschieden werden kann, glauben wir doch annehmen zu können, dass die fermentaktive Schicht der Zelle selbst bzw. ihrer Plasmamembran zugehört. Eine Bindesubstanz kann nämlich an lebenden Zellen im Phasenkontrastmikroskop nicht nachgewiesen werden. Auch die Ablösung von Teilen der Zelloberfläche infolge Trypsin- oder Versenewirkung kann unserer Meinung nach nicht als Argument dafür verwendet werden, dass es sich um eine der eigentlichen Zelloberfläche aufgelagerte Bindeschicht handelt. Denn bei der phasenkontrastmikroskopischen Untersuchung dieses Ablösungsvorganges sind im Zusammenhang mit den am Glas haftenden Teilen der Zelloberfläche Granula zu beobachten, die aus den äusseren Zonen des Cytoplasmas stammen müssen. Dass zum mindesten Versene, offenbar durch Bindung von Calcium, zu einer Desintegration der Zelloberfläche führt, hat Coman [3] in einer elektronenmikroskopischen Untersuchung an der Leber nach Versene-Perfusion bewiesen. Der Annahme, dass äussere Schichten aus einer solideren gelartigen Zelloberfläche herausgelöst werden können, ohne dass die Zellen einen irreparablen Schaden erleiden, widerspricht nichts. Wechsel des Sol $\rightleftharpoons$ Gelzustandes im Bereich der Zelloberfläche, die u.a. auch Zollinger [18] zur Erklärung der Vorgänge bei der Ablösung von Blasen von der Zelloberfläche im Laufe der Potocytose heranzieht, machen es verständlich, dass die Lücken der Zelloberfläche sofort durch den Übergang angrenzender Cytoplasmaschichten in den Gelzustand geschlossen werden können.

ZUSAMMENFASSUNG

An in vitro gezüchteten Zellen des HeLa-Epidermoidcarcinoms kann mit histochemischen Methoden alkalische Phosphatase nachgewiesen und genauer

kalisiert werden. Zellen des morphologisch sehr ähnlichen Epidermoidarcinoms Stamm A sind frei von nachweisbarer alkalischer Phosphatase.

Die Calcium-Cobalt-Methode bringt in HeLa-Zellkulturen das Ferment in rster Linie an Zellgrenzen und in geringerem Masse im Cytoplasma zur arstellung. Mit der Azokupplungsmethode findet sich eine stärkere Aktivität n Cytoplasma und an den Falten der Zelloberfläche, die an der lebenden elle durch ihre lebhaften Bewegungsvorgänge gekennzeichnet sind.

Die mit den beiden Methoden an den HeLa-Zellen gefundene Aktivität er alkalischen Phosphatase gehört einer Schicht der Zelloberfläche an, die ir den Zellzusammenhalt und für das Haften der Zellen an der Glasunterlage on Bedeutung ist. Teile der fermenttragenden Schicht der Zelloberfläche önnen durch eine Einwirkung von Trypsin oder Versene, die die Lebensihigkeit der Zellen nicht beeinträchtigt, abgelöst werden. Wenn solche von en Zellen abgelöste Teile der Zelloberfläche an der Glasunterlage haften leiben, lässt sich in ihnen alkalische Phosphatase nachweisen. Es wird ermutet, dass die fermenthaltige Schicht einen Bestandteil der Zelloberäche selbst, d.h. der Plasmamembran, und nicht eine extrazelluläre Bindeubstanz darstellt.

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THE GROWTH OF SINGLE CELLS

II. *SACCHAROMYCES CEREVISIAE*

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Received November 30, 1957

The first paper of this series [6] described measurements with an interference microscope of the growth in dry mass and volume of single cells of a fission yeast, *Schizosaccharomyces pombe*. An interesting and somewhat surprising fact that emerged from this work was that the dry mass growth of a single cell was linear during the cell's life cycle. In other words, once a cell had been formed by the division of its mother, its rate of dry mass increase remained constant until it in turn divided. The present paper describes a similar set of measurements on a budding yeast, *Saccharomyces cerevisiae*. The results show that the budding yeast cell also has a constant rate of increase of dry mass during most of its life cycle, even though its volume growth and its method of cell multiplication are different from those of the fission yeast.

MATERIAL AND METHOD

The budding yeast that was used in this work was *Saccharomyces cerevisiae* var. *ellipsoideus* (N.C.Y.C. 125). This variety was chosen because it has smaller cells than the ordinary *S. cerevisiae*, and therefore a lower retardation in the interference microscope. Stock cultures were kept in 2 per cent w/v "Oxoid" Wort Broth.

The method of mounting and measuring the growing yeast cells was similar to that used for the fission yeast [6]. Very briefly, this consisted in growing the yeast cells in a gelled medium containing bacteriological gelatin and Wort Broth. The dry mass of a single growing cell was measured every seven and a half minutes by means of an integration method with a Baker interference microscope [7]. A photograph was also taken in order to calculate the cell volume.

There were some minor differences between the technique used in these experiments and that used for the fission yeast. Owing to the greater thickness of the budding yeast cells, a more concentrated medium was needed in order to reduce their maximum retardation to 45°. The medium was made up with an initial concentration of 22.5 per cent bacteriological gelatin and 2 per cent Wort Broth, but the final measured concentration of solids was 0.204 g/ml. The yeast was grown overnight before being mounted at 30°C, and the cells were not sedimented during mounting. During the growth measurements the cells were kept at 23°C by a warm box round the microscope which was preferable to a warm stage. The volume of individual cells was calculated from the following formula:

$$V = \pi a b^2/6$$

where a is the major axis of the cell, and b is the minor axis. This formula assumes that the cell is a prolate ellipsoid of revolution (round the major axis).

RESULTS

Before the results are presented, it is necessary to give a brief description of the method of growth of this budding yeast. A budding cycle starts with the appearance of a bud at some point on the periphery of a mother cell. This bud grows until it reaches the size of its mother cell, but during this time there is no change in the dimensions of the mother. The second cycle starts with the production of two buds; one from the old mother cell, and the other from the cell that was a bud in the preceding cycle but has now become a mother. During the second cycle and the succeeding ones, the mother cells remain unchanged in size; this is an important point, and worth emphasising. A yeast cell has only one period of volume growth -while it is being produced as a bud. Thereafter it does not change in size though it continues to produce buds.

After a number of pilot runs, sixteen main experiments were carried out, each starting with a mother cell and a bud. Measurements were made through the rest of this cycle, and through the next one also where there were two mother cells and two buds. It was usually impossible to continue the measurements into the third cycle, since the cells began to lie over each other. The results from one experiment are shown in Fig. 1 as curves of dry mass, volume and concentration plotted against time. The main features of these curves were present in all the other growth curves. The dry mass growth curve is substantially linear for the major part of each budding cycle, but the rate of growth of dry mass for the first cycle (one mother cell and one bud) changes quite sharply to the new and higher rate for the second cycle (two cells and two buds) about 20 minutes after the appearance of the two new buds. The volume growth curve is sigmoid; the rate of volume growth of a bud increases for about the first third of a cycle, then stays constant for the second third, and in the last third decreases. The concentration curve cycles up and down with a maximum about 20 minutes after budding, and a minimum about 40 minutes before budding.

The volume increase in the growth curves is caused only by the growth of the buds, since the volume of mother cells remained unchanged. The changes in dry mass and concentration, however, occur in the combined system of buds plus mother cells. This method of measuring dry mass with the interference microscope takes into account everything that lies within the field aperture, and it is impossible to determine how much of the dry mass changes are due to the mother cells and how much to the buds. When observed visually, however, the dry mass of the mother cells appeared to increase towards the end of each cycle, and they showed a higher retardation at this stage than in the middle of a cycle.

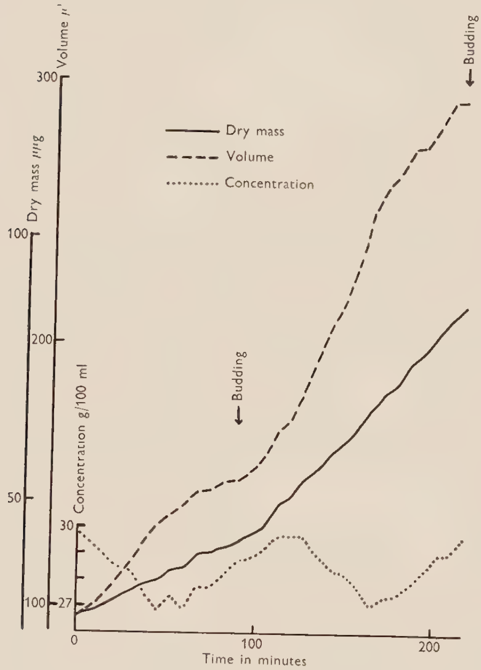

Fig. 1.—Growth curves. One mother cell and one bud up to 90 minutes; after that, two mother cells and two buds. The arrows mark the first appearance of buds at the beginning of each cycle.

As with the fission yeast, it was necessary to do a statistical analysis of the dry mass growth curves in order to prove the important point of a linear growth rate in each cycle. The experimental curves were divided into two sections corresponding to the two budding cycles. The first section, for one mother cell and one bud, ran from the start of the experiment to the time of the first budding. The second section, for two mother cells and two buds, ran from 30 minutes after the first budding to the second budding. A quadratic curve was then fitted to each section by the method of orthogonal polynomials, and two tests for linearity were applied. First, each section was taken

TABLE I. *Data from growth curves.*

Saccharomyces cerevisiae v. *ellipsoideus.* Wort Broth gelatin. 23° C.

	Units	Mean	Standard deviation (±)	n
ry mass				
No. of points in each section of growth curve.		12 6	2.8	25
Slope: 1 cell + 1 bud.	$\mu\mu$g/min	0.169	0.038	10
Slope: 2 cells + 2 buds.	$\mu\mu$g/min	0.317	0.057	10
Increase of slope: 2 cells + 2 buds/1 cell + 1 bud.		1.99	0.24	8
Mass of each cell before budding: 2 cells.	$\mu\mu$g	19.9	2.05	13
Mass of each cell before budding: 4 cells.	$\mu\mu$g	19.1	2.03	11
Meeting point of fitted curves: time after budding.	mins	20.2	3.9	8
olume and dimensions				
Volume of each cell before budding: 2 cells.	μ^3	65.7	6.9	13
Volume of each cell before budding: 4 cells.	μ^3	64.2	6.9	11
Dimensions of cell before budding: major axis.	μ	6.0	0.6	14
Dimensions of cell before budding: minor axis.	μ	4.4	0.3	14
ncentration and time				
Min. concentration: 1 cell + 1 bud.	g/100 ml	28.0	1.1	13
Time of min. concentration before budding: 1 cell + 1 bud.	mins	38.6	8.3	13
Min. concentration: 2 cells + 2 buds.	g/100 ml	27.9	1.1	10
Time of min. concentration before budding: 2 cells + 2 buds.	mins	39.4	6.5	10
Max. concentration: 2 cells + 2 buds.	g/100 ml	31.5	2.0	12
Time of max. concentration after budding: 2 cells + 2 buds.	mins	19.3	11.1	12
Generation time: between successive budding.	mins	132	13	10

parately and the significance of the quadratic variance was tested against e residual variance. In 17 out of 25 sections the quadratic term was not gnificant for P 0.05, so the sections fitted a line better than a quadratic rve. Of the remaining 8 sections, 4 showed a significant upward curvature d 4 a significant downward curvature. Secondly, a combined test of significance was made on the sum of the quadratic coefficients weighted by the ciprocals of their residual variances. This was not significant for all 25 ctions ($P = 0.49$), showing that there was no significant tendency of curvare in either direction. The reasonable conclusion from these tests is that e growth in dry mass is linear for the major part of each budding cycle.

A summary of the data from the fitted curves and from the experimental curves is given in Table I. The dry mass slope is the linear coefficient of the fitted curves. The meeting point of the fitted curves gives the best estimate of the point where the rate of mass growth increases sharply after budding. The other data in Table I are taken from the experimental curves.

Two anomalous growth curves have not been included in the data in Table I. In both cases, the mother cell suddently began to grow in volume after it had produced an almost full-sized bud. There was also a sharp increase (about 1.8 ×) in the rate of mass growth at the same time. This abnormal growth continued for about 100 minutes until budding eventually took place. In both cases the cells died shortly after the end of the experiment.

DISCUSSION

As with the fission yeast [6], the most interesting aspect of these results is that the dry mass growth curve is not exponential but is rather a series of straight lines with sudden increases in rate at each budding cycle. At first sight, one might regard the appearance of a bud as the start of a new generation in a budding yeast. If so, there would be a discrepancy between the results with the budding yeast and those with the fission yeast. In the budding yeast, the change in rate of mass growth takes place about 20 minutes after the appearance of the new bud. But in the fission yeast, this change in rate happens at or before cleavage. This discrepancy, however, is more apparent than real since it rests on the false assumption that a generation in a budding yeast starts with the appearance of a new bud. The best definition of the start of a new generation is the point when a new cell is separated physiologically either from its mother cell (in budding) or from its sister cell (in fission). The exact point at which a cell wall is laid down between a mother cell and a bud is unknown, but it is probably in the second half of the budding cycle when the bud is approaching its mature size. Electron microscope photographs show a cell wall between bud and mother in a mature bud but not in a young bud [1]. Also granules can be seen passing from a mother cell into a young bud [2]. If then we regard the latter part of the budding cycle as the starting point of a new generation in a budding yeast, a number of facts fit into place. The apparent discrepancy between the budding and the fission yeast vanishes, since in both cases the change in rate of mass growth occurs at or before the start of a new generation. Secondly, the splitting of nuclear material happens at the normal place in a cell life cycle—towards the end of a generation. Mitosis, or its equivalent in a yeast, takes place at he beginning of a budding cycle and new nuclear material moves into the young bud [3, 4]. Thirdly, the duplication of DNA occurs in interphase. Ogur, Minckler and McClary [8] have shown that the full complement of DNA for the new generation is synthesised by the time that the buds appear.

This concept of generations in a budding yeast means that the "cell" of each generation starts by being either a nearly mature bud or a mother, and finishes by being a combined system of bud plus mother. The experimental results suggest very strongly that every cell has a constant rate of growth in dry mass throughout its generation (or at any rate until very near the end), but there is one gap in the argument. A new generation starts when a bud separates from its mother by the formation of a cell wall between them, and both cells should then start to grow. But, although the bud continues to grow in size, there is no change in the size of the mother until the new cycle of buds begins. The most likely solution of this difficulty is that the mother cell increases in dry mass and concentration even though its volume remains unchanged. There is observational evidence that this occurs (p. 216), but no measurements. The experimental curves only give the results for two or more cells and it is impossible to separate the contributions of each cell to the total growth curves. Nevertheless, I think it is very likely that the rate of growth of dry mass of a mother before budding is the same as that of the combined system of mother plus bud after budding. It is worth pointing out that the increase in internal hydrostatic pressure which probably follows the increase in concentration in a mother cell may well be the immediate cause of bud formation.

In normal logarithmic growth, each generation of cells should grow at the same rate as the preceding one. This did not happen with the fission yeast and the cell growth rate slowed down at each generation, presumably due to unfavourable conditions on the microscope slide. No direct evidence could therefore be produced that linear mass growth occurred during normal logarithmic growth, though indirect arguments made it seem very likely. The situation with the budding yeast, however, is more satisfactory. Table I shows that the total growth rate in the second cycle was almost exactly double the rate in the first cycle, so the growth rate per cell remained unchanged. Also the mean dry mass and volume of individual cells before budding showed very little change from one cycle to the next. The cells were therefore growing logarithmically during the experiments.

The implications of a linear growth rate have been discussed both in the earlier paper on the fission yeast [6] and in a recent review by Swann [9]. All that needs to be said here is that a linear growth rate suggests that synthesis is controlled by synthetic centres, perhaps microsomal particles, which remain constant in number or effect throughout a cell life cycle and only double at division, when they partition themselves between the two daughter cells. In the case of a budding yeast, the centres would double at the young bud stage and half of them would then move into the bud before it finally separated from the mother. Although the budding yeast and the fission yeast belong to the same group of micro-organisms, they have different methods of division and different shapes of volume growth curve. The fact that linear dry mass growth occurs in both these cells despite their differences suggests that it may be a common feature in other cells.

The first published measurements of the volume growth of budding yeast were made by Bayne-Jones and Adolph [2]. Their curves of the volume increase of single cells are substantially the same as those presented in this paper. They found an increasing rate of volume growth for the first 20 minutes after the appearance of the bud, then linear growth for the next 50 minutes, and finally a declining rate of growth for the last 20 minutes. The only difference between their results and mine was that their period of linear growth occupied a larger proportion of the cycle. This might be due to the use of a different variety of *S. cerevisiae* and a higher growing temperature (37°C).

Lindegren and Haddad [5] also measured the volume growth of individual budding yeasts. Their results are not unlike those of Bayne-Jones and Adolph, though they emphasise the linear parts of the growth curve. They argue that the mass growth must also be linear during the linear volume growth, since they found no change in the specific gravity of growing yeast cells using Stokes Law. The results from the interference microscope measurements, however, show that there are changes in concentration and therefore specific gravity during growth though these are probably too small to be detected by the relatively insensitive method used by Lindegren and Haddad. Their conclusion of linear mass growth is in fact correct though it extends beyond the period of linear volume growth.

It is worth drawing attention to the two anomalous growth curves which were mentioned earlier (p. 216). These are interesting because one possible interpretation is that the mechanism for increasing the mass growth rate had become dissociated from the budding mechanism. Normally, these are closely linked since the growth rate increase takes place at a fixed time after budding, but in these two cells, the rate increase took place at an abnormal point in the growth cycle—before budding. In this case, however, it seems that the penalty for dissociating growth from division is death.

SUMMARY

1. Measurements were made of the growth in dry mass and volume of ingle cells of the budding yeast *Saccharomyces cerevisiae.* Dry mass was neasured by an integration method with an interference microscope, and olume was measured from photographs.

2. The growth in dry mass was linear for each budding cycle. The comined system of mother cell plus bud grows in dry mass at a constant rate rom 20 minutes after the appearance of the bud until 20 minutes after the ppearance of the next pair of buds. It is concluded that dry mass growth s linear for most of each cell generation. This result is similar to what was ound earlier in the fission yeast *Schizosaccharomyces pombe* and lends support to the hypothesis that synthesis is controlled by centres or particles, erhaps microsomal, which remain constant until division, when they double nd partition themselves between two daughter cells.

3. The curve for volume growth is approximately sigmoid.

4. The cell concentration fluctuates cyclically with a maximum about 20 inutes after budding, and a minimum about 40 minutes before budding.

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AN ELECTRON MICROSCOPE STUDY OF DIFFERENT SAMPLES OF DNA

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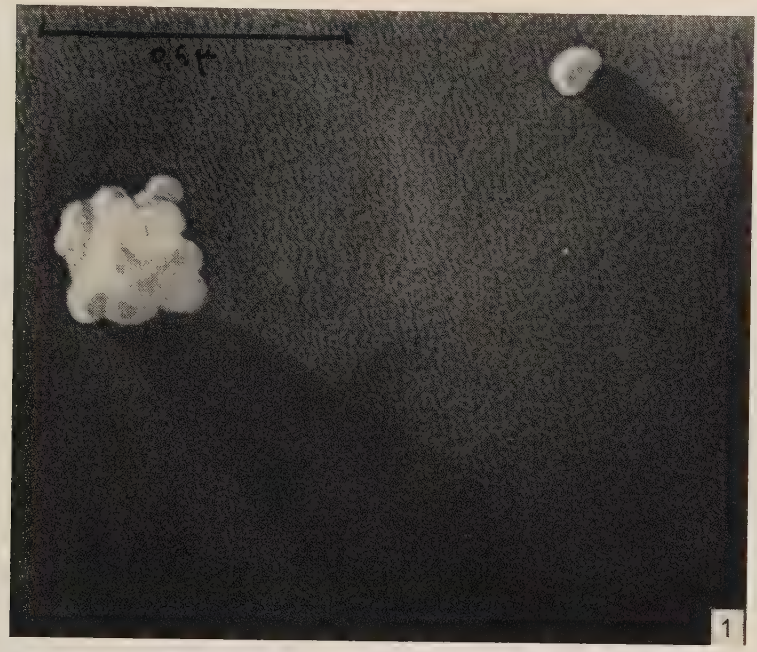
Received November 30, 1957

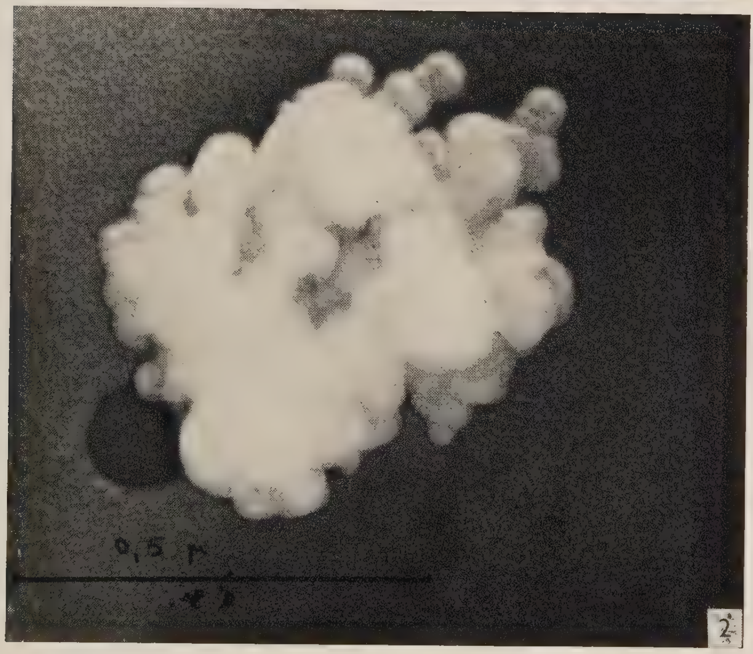
A NUMBER of authors have studied the aspect of desoxyribonucleic acid at the electron microscopic level. Most of them [2, 3, 5, 6, 9, 10, 11] obtained electron microphotographs showing the DNA as long filaments varying in diameter—the thinner ones having a diameter of 20 Å. A few authors [1, 4, 2] saw the DNA in a globular form, but not much attention was paid to this since this form seems to be in disagreement with the physical data, which indicate that the DNA in solution must rather be in the form of more or less well oriented filaments.

We have recently described a method of preparation of highly polymerised DNA in the form of an extremely fine powder [8]. The nucleoprotein extracted from calf thymus by saline solution was deproteinised by prolonged contact with saturated solution of sodium chloride following the method of Signer and Schwander [7]. After a complete removal of salt by a number of dissolutions in distilled water followed by reprecipitations in alcohol, this material becomes soluble in alcohol of high titer. When a solution of DNA is obtained in isopropylic alcohol at 75°, the addition of a small quantity of sodium chloride to this solution results in the formation of an extremely fine precipitate which is collected and dried with dioxane.

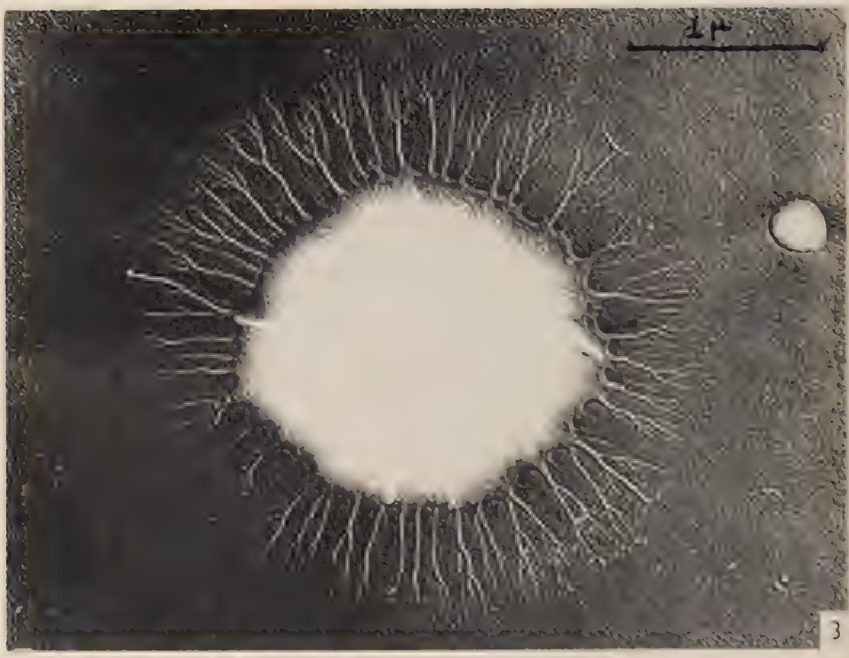
The chemical analysis of this powder gives data which are typical of DNA: 8.82 per cent of phosphorus, 15.6 per cent of nitrogen—the ratio N/P being 1.76. The molecular mass determined by light scattering is about 8 millions. This form can be readily examined with the electron microscope after shadowing with chromium. We obtained in this way images of spheres of about 300 to 400 Å diameter or aggregates of spheres (Fig. 1 and 2).

It is possible to realise a gradual hydration of this powder by transferring it from 0° in a dry atmosphere to 25° in a wet atmosphere for a few minutes. The preparation is then dried in vacuum and shadowed. The pictures thus obtained (Figs. 3, 4 and 5) show the appearance, at the periphery of the aggregates, of a great number of filaments radiating all around—the thinnest





gs. 1 and 2.—Sodium desoxyribose nucleate, pulverulent preparation chromium shadowed
g. 1, × 80,000; Fig. 2, × 110,000.



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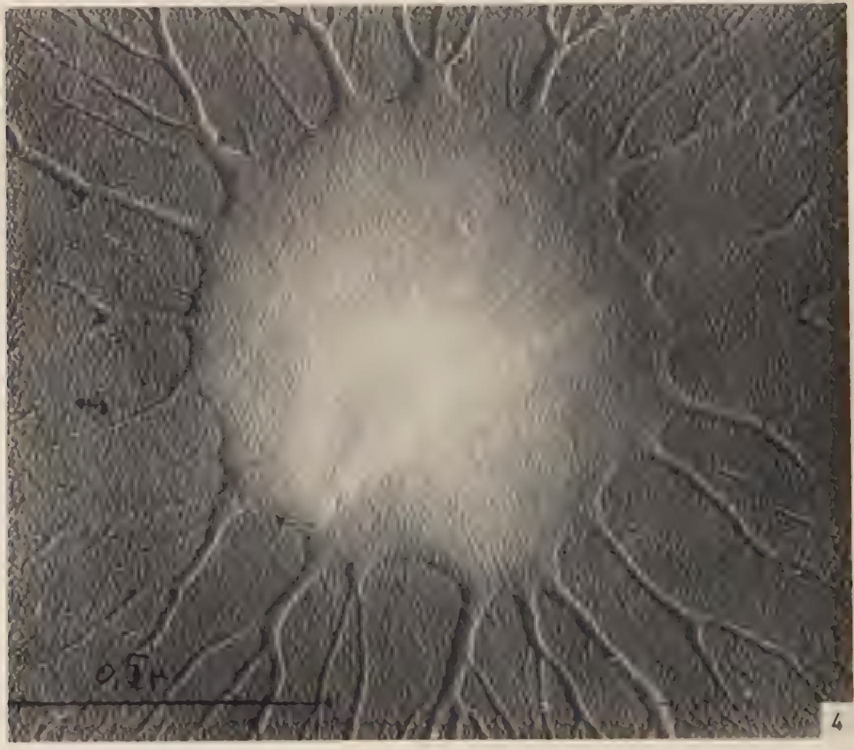

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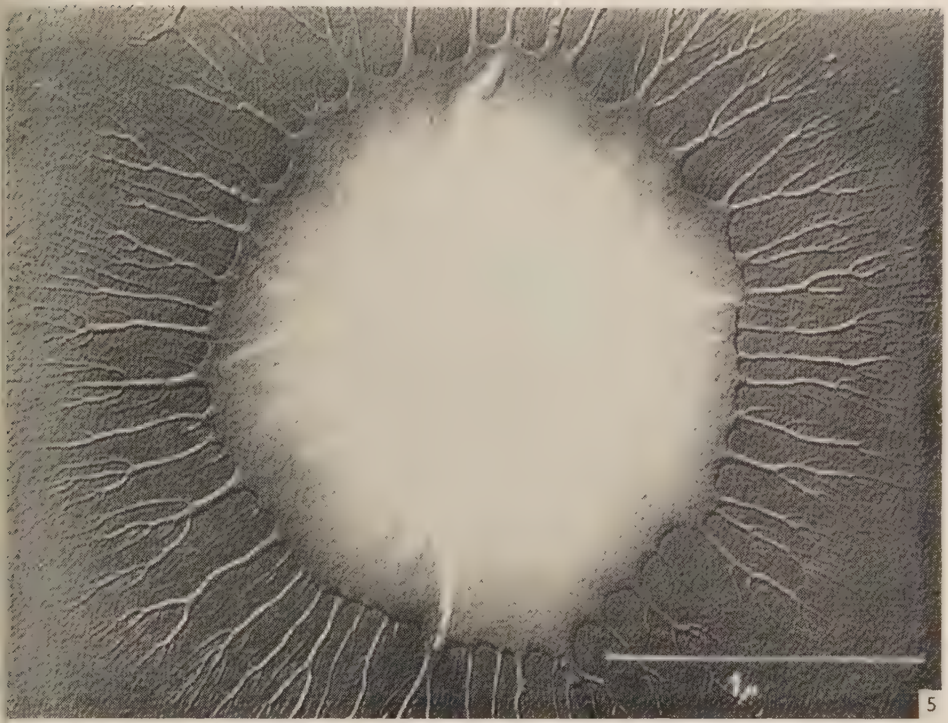

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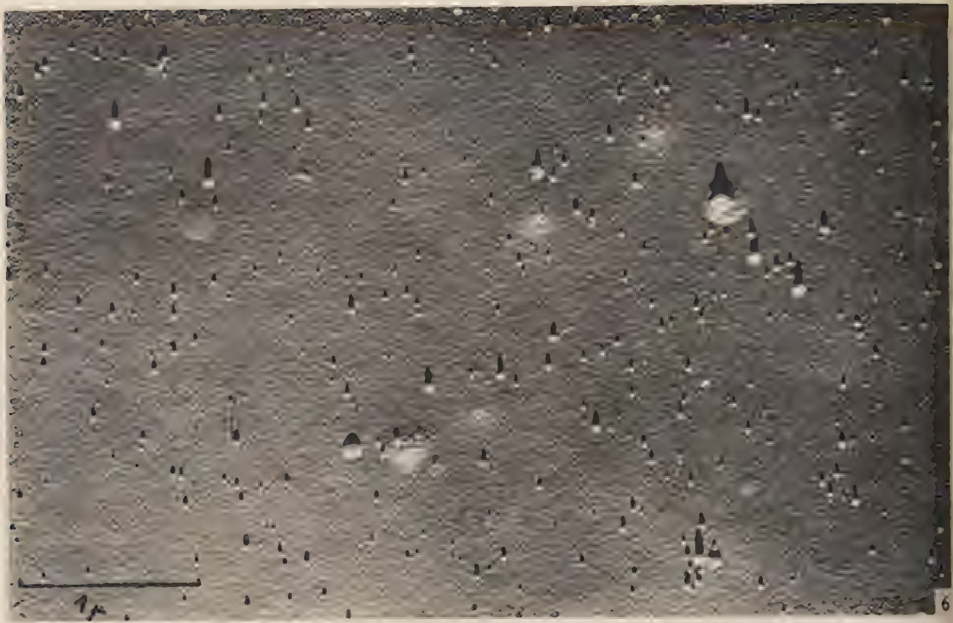
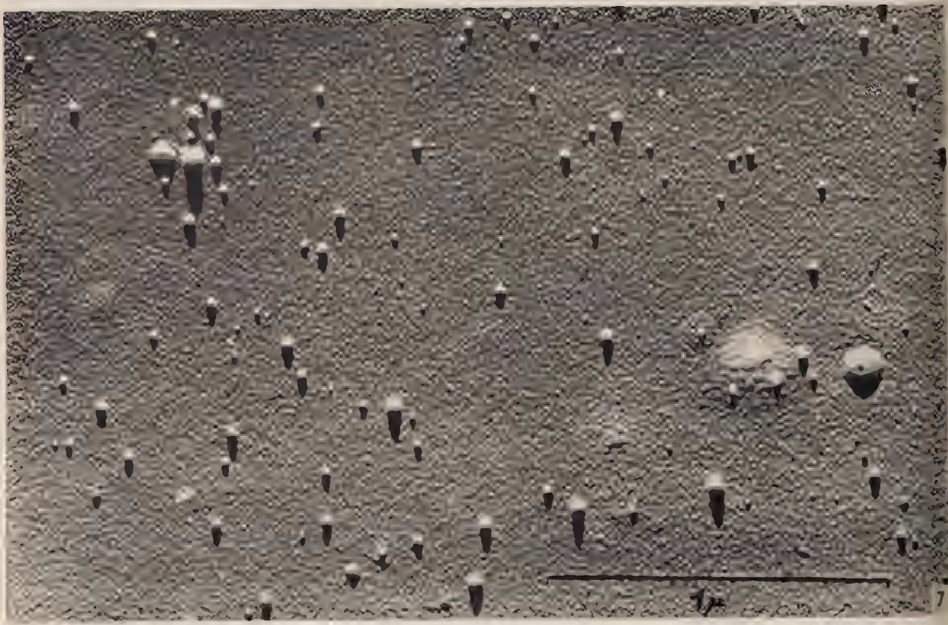
gs. 3, 4 and 5.—Sodium desoxyribose nucleate, pulverulent form as Figs. 1 and 2 after gradual dration—chromium shadowed. Fig. 3, × 25,000; Fig. 4, × 87,000; Fig. 5 × 42,000.

these filaments have a diameter of 20 Å. We can see here the passage from e globular form to the filamentous one.

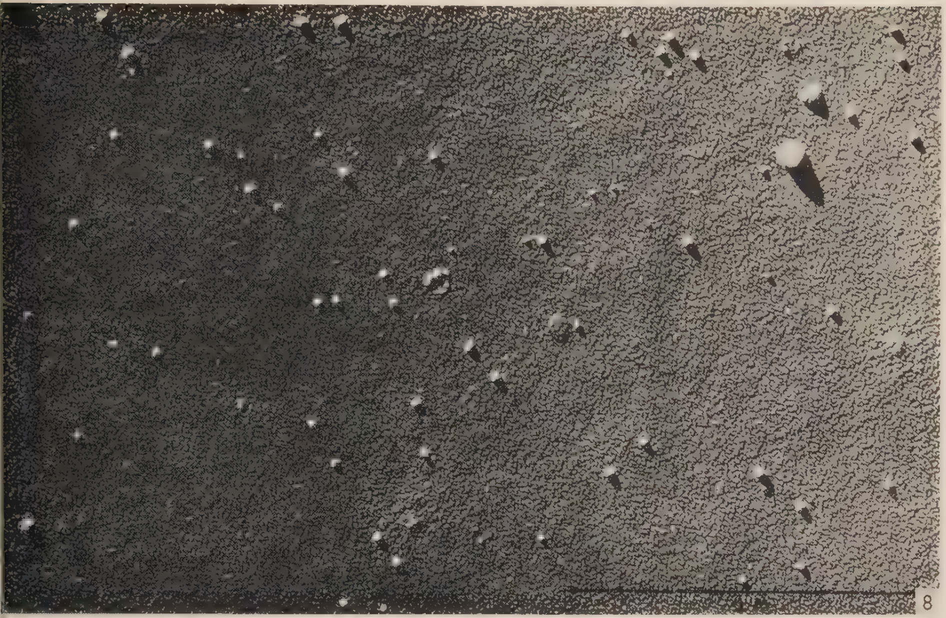
Beside this powder form of DNA, we also studied solutions of DNA in istilled water—the dilution was of 1 to 4 mg DNA per liter. These solutions ere dialysed thoroughly in order to remove all residue of salt, and a small op of solution was put on the holder and dried in vacuum. After shadowing, e obtained the pictures represented in Figs. 6, 7 and 8. The dimensions of ese globular forms range from about 100 to 400 Å.

DNA dissolved in water and absolutely devoid of salt, appears in a globular rm. Is it possible to consider these forms, or, at least, the smallest of them, individual molecules? It is difficult to answer this question. We tried to rovoke an uncoiling of these globular forms: the addition of a small quany of sodium chloride to the solution of DNA (3 cc NaCl, 0.14 *M*/liter), efore the deposition of a drop on the holder, dessication and shadowing

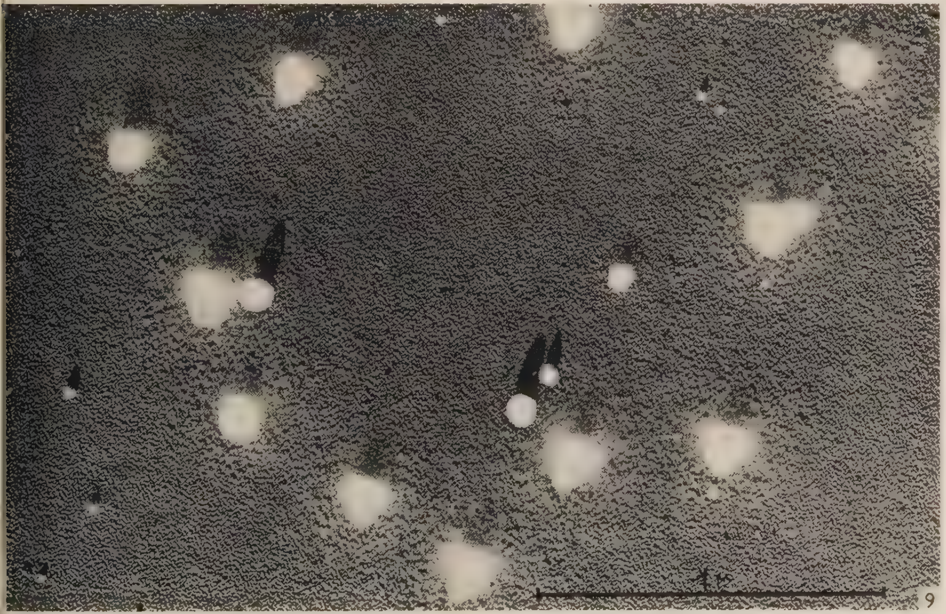




Figs. 6 and 7.—Sodium desoxyribose nucleate (Preparation CV 49, R. Vendrely), dialysed solution, dried on microscope grid—chromium shadowed. Fig. 6, × 24,000; Fig. 7, × 45,000.



ig. 8.—Sodium desoxyribose nucleate (Preparation S VIII, R. Signer), dialysed solution, dried n microscope grid—chromium shadowed. ×45,000.



g. 9.—Sodium desoxyribose nucleate dialysed solution added with NaCl, then dried on the icroscope grid—chromium shadowed. ×45,000.

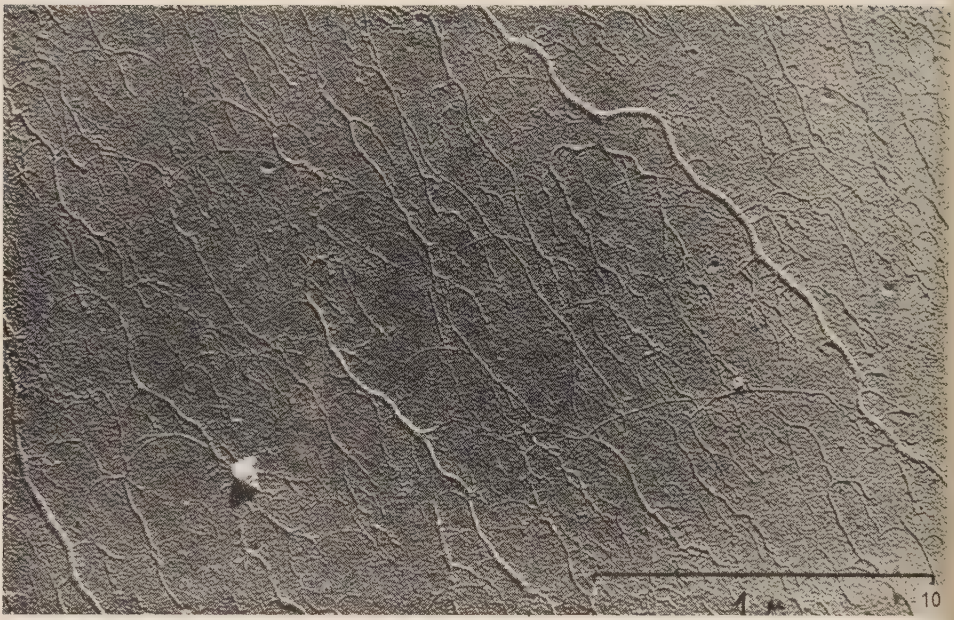


Fig. 10.—Sodium desoxyribose nucleate (Preparation C V 49, R. Vendrely), fibers impregnated with NaCl before dissolution. The solution was dried on the microscrope grid and chromium shadowed. ×45,000.

produced the aspect of the figure [9]; the globules have lost their sharp outlines and begin to disaggregate.

But if we put several mg of DNA in a small volume of a saturated solution of sodium chloride (1 or 2 cm), allow it to remain in contact overnight, and then dilute to obtain a final concentration of 1 mg/l, a total uncoiling of the globules is realised and the DNA appears as long fibers (Fig. 10).

It thus seems that the presence of salt is necessary to maintain the fibers of DNA in an extended state. The development of fibers from the powder form of DNA by simple hydration is made possible by the presence of salt retained in the precipitate.

On the other hand we have seen that when the sodium chloride is completely removed from DNA by a thorough dialysis, this DNA appears in a globular form at the electron microscopic level.

The behavior of the DNA molecules in solution seems thus to depend very much on the quantity of impregnating salt after they have been precipitated. When the DNA is put back in water, this residual salt makes possible its uncoiling. If all the salt has been removed, the molecules remain tightly coiled.

SUMMARY

The different aspects presented in the electron miscroscope by the molecules f DNA (globular forms and filamentous forms) could be explained by the egree of salification of the product: a more or less complete uncoiling of the olecule or, on the contrary, a tight coiling would occur depending on the resence or absence of salt.

The authors are indebted to Miss S. Haasser and Miss D. Hiltenbrand for technical ssistance.

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BRIEF NOTES

THE DEVELOPMENT OF POLYSOMATY IN RAT LIVER[1]

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Received May 13, 1958

Many animal tissues are known to be composed of cells containing nuclei of various degrees of polyploidy, correlated with corresponding variations in nuclear volume and DNA content (cf. Swift [8]). Such polysomatic patterns have been studied extensively in rodent liver where they are usually quite pronounced. Fusion of nuclear material in binucleate cells during interphase or mitosis [1] and endomitosis (e.g., Swartz [7]) are the principal mechanisms invoked by various authors to explain the development of polysomaty (see discussion by Wilson and Leduc [9]). In recent years it has also become evident that the polysomatic growth pattern can be influenced by various hormonal [2, 5] and environmental [4] factors. We present herein a brief account of the methods we have employed to study polysomaty, and a discussion of some general aspects of the normal development of polysomaty in rat liver.

Livers of rats of the Long-Evans strain were fixed in acetic-alcohol and embedded in paraffin; sections were stained by a combined Feulgen-PAS procedure: glycogen was first removed by digestion with saliva and the sections were then hydrolyzed according to the Feulgen method, washed and subsequently oxidized with periodic acid prior to exposure to the Schiff reagent. Both nuclei and cell membranes are stained by this method. Nuclear ploidy can then be scored visually according to the combined criteria of size and staining intensity, and the stained cell outlines facilitate the detection of binucleate cells.

In order to establish adequate counting procedures cells were scored under oil immersion in sections of various thicknesses, along single lines or in strips of tissue and in different parts of the same liver; counts were also repeated by the same and by different observers. An analysis of these counts, made under the direction of Dr. Elizabeth Scott,[3] Department of Statistics, University of California, disclosed that many difficulties are involved in the collection of such data, including considerable variability in reproducibility, differences among animals of the same age and sex, and influence of conditions under which the counts are obtained. For instance, counting along a line discriminates against the detection of small cells and nuclei; moreover, section thickness influences markedly the frequency count of binucleate cells, since two nuclei may become separated during the cutting process (see also Harrison [3]). Counting is, therefore, now done by a single observer on batches of ssues processed together under identical conditions; fairly wide counting strips 6.4 μ) defined by two parallel lines in an ocular disc, and thick sections (15 μ) are sed. At least 1000 cells per liver sample are scored.

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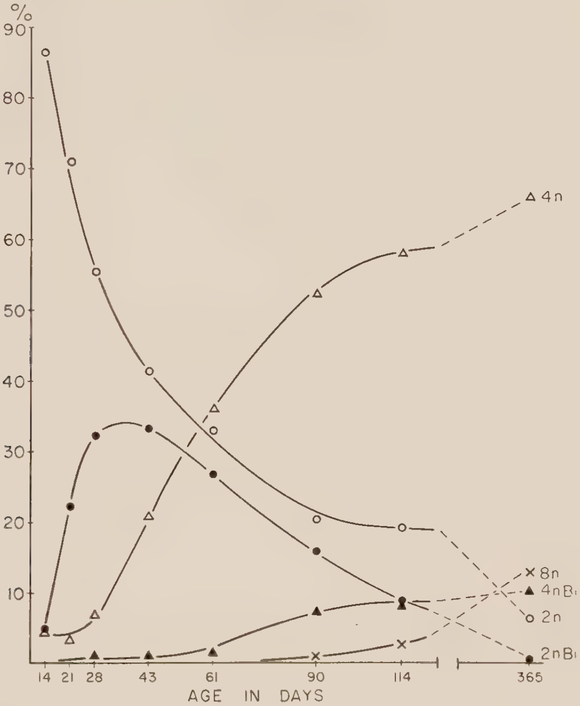
[1] Supported by Cancer Research Funds of the University of California.

[2] With the able technical assistance of Lowell Wilson, John Pun, and Caroline Schooley.

[3] We are indebted to Dr. Scott for carrying out these statistical analyses.



g. 1.—Cell composition of rat ver parenchyma at various ages. evelopment of polyploidy in rat ver. $2n$, diploid; $2n$ bi, cells with diploid nuclei; $4n$, tetraploid; etc.

A study of the development of the complete ploidy pattern in female rats up to ne year of age was made, and the results are shown in Fig. 1, where data obtained n three animals in each age group are averaged. It can be seen that the liver cell opulation of rats shortly after birth is predominantly diploid and that cells of in- easing degrees of ploidy appear in sequence and rise in frequency with age. The radual shift towards increased polyploidy is accompanied by the appearance of inucleate cells interposed between successive classes of mononucleate polyploid ells. (Such a sequence is consistent with the view that binucleate cells contribute to ne formation of higher polyploids, cf. Himes *et al.* [6]). Only the qualitative aspect these results should be emphasized. The absolute frequencies of cell types at a ven age are bound to vary in rats of different strains or in animals raised under ifferent conditions, but the overall trend of changes indicated here appears to be of eneral significance.

These results indicate that in any experimental study of polysomaty the dynamic ature of the liver picture must be kept in mind. Experimental alterations of the olysomatic pattern should be most easy to detect in animals up to three months of ge, since the most marked changes in the normal picture occur during this period.

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LIVER REGENERATION AND HEPATIC POLYPLOIDY IN THE HYPOPHYSECTOMIZED RAT[1]

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Received May 13, 1958

It has been known for some time that the cells of many adult mammalian livers show polyploidy. In the present paper an examination is made of some factors involved in the hormonal control of polyploidy in rat liver (cf. preceding note [1]).

As shown by Di Stefano *et al.* [2] in rats the progression towards higher levels of ploidy in the liver can be arrested by hypophysectomy; furthermore, the polysomatic pattern is essentially absent in livers of hereditary pituitary dwarf mice [3, 5] but its appearance can be induced by treatment with pituitary preparations more or less pure with respect to growth hormone. Similarly, the progression toward higher levels of ploidy can be reinstated in the hypophysectomized rat by means of such preparations [2].

The present experiments were undertaken to determine whether growth hormone is a *sine qua non* for the progressive development of the polysomatic growth pattern. As mentioned above, hypophysectomy is known to arrest the further increase of polyploidy with age; it is also known that liver restoration following hepatectomy [6] and CCl_4 poisoning [4] in otherwise intact animals is accompanied by an accelerated shift towards higher levels of ploidy. We have now determined the cell population in the regenerated livers of rats subjected first to hypophysectomy and subsequently to partial hepatectomy.

In two preliminary experiments male rats of the Long-Evans strain were hypo-

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[1] Supported by Cancer Research Funds of the University of California.

[2] With the expert technical assistance of Charles Jordan and Norma O. Goldstein.

ABLE I. *Distribution of binucleate and polyploid cells in regenerating liver of the hypophysectomized rat.*

			Percentage of cell types				
Group[a]	No. of animals	Lobe[b]	$2n$	$2n$ Binucleate	$4n$	$4n$ Binucleate	$8n$
H at 21 d, S at 28 d	4	R	72.2 (63.1–77.6)[c]	21.9 (16.3–30.5)	4.5 (3.1–5.6)	0.3 (0.1–0.5)	0.0
H at 21 d, Hepat at 28 d, S at 42 d	3	R	54.8 (53.4–57.0)	10.7 (9.6–11.6)	31.5 (28.8–34.7)	1.2 (0.8–1.5)	0.4 (0.3–0.5)
H at 42 d, S at 49 d	5	R	36.6 (28.1–47.0)	21.9 (18.5–25.7)	37.4 (24.7–48.5)	2.2 (1.5–2.9)	0.2 (0.1–0.4)
H at 42 d, Hepat at 49 d, S at 63 d	8	R	18.4 (11.2–25.0)	2.7 (0.5–4.8)	69.8 (63.1–77.9)	1.7 (0.7–2.9)	4.7 (1.8–10.2)
H at 21 d, S at 28 or 42 d	7	R	81.2 ± 2.2[d]	13.5 ± 1.9	3.8 ± 0.6	0.1	0.0
H at 21 d, Hepat at 28 d, S 42 d	9	L at 28 d	77.5 ± 1.4	15.5 ± 1.6	4.6 ± 0.7	0.2	0.0
		R at 42 d	59.7 ± 2.3 $p<0.001$[e]	9.6 ± 1.2 $p=0.01$	28.4 ± 1.9 $p<0.001$	0.8 ± 0.2 $p<0.02$	0.4 ± 0.1 $p<0.001$
Normals, S at 42 d	6	R	58.6 ± 3.1 $p<0.001$[f]	14.8 ± 1.8 $p>0.05$	25.4 ± 4.2 $p<0.001$	0.7 ± 0.1 $p<0.001$	0.2 ± 0.1 $p>0.05$

[a] H, hypophysectomized; Hepat, partially hepatectomized; S, sacrificed.
[b] R, right lateral; L, left lateral.
[c] Range.
[d] Mean ± standard error.
[e] "p" values for R at 42 d vs. L at 28 d (line 7 vs. line 6).
[f] "p" values for Normals vs. H at 21 d, S at 28 or 42 d (line 8 vs. line 5).

hysectomized either at 21 or 42 days of age; one week later a number of these nimals were hepatectomized and after two more weeks they were sacrificed. Right teral lobes were removed, fixed, sectioned and stained together; the nuclei of at ast 1000 cells per animal were then scored by previously described methods [1]. he results of these experiments, incomplete due to the loss of tissues from terminal ntrol animals, are given in the upper half of Table I. It may be seen that the ploidy istribution arrested by hypophysectomy at 42 days (line 3, Table I) is much more dvanced (fewer $2n$, and more $4n$ and $8n$) than that arrested at the 21-day age level ine 1, Table I). Furthermore, the regenerated livers of both groups (lines 2 and 4, able I) exhibit a marked shift towards higher levels of ploidy. Although an unambiguous evaluation of these results, considered by themselves, cannot be made due to the loss of the appropriate controls, accumulated evidence suggests that the data for such a control group would not have differed markedly from the reported control values. Thus, Di Stefano *et al.* [2] have shown that hypophysectomy halts the development of ploidy, and in unpublished experiments we have found that in animals 100 days posthypophysectomy, the ploidy pattern had progressed an equivalent of at most one week's further growth after the time of hypophysectomy. This is also borne out by the results of Experiment 3, reported below.

More extensive results of a third experiment are given in the lower half of Table I. Two groups of animals were used, one intact control group sacrificed at 42 days and a group hypophysectomized at 21 days. From the latter, two animals were sacrificed at 28 and five at 42 days; the remainder of the hypophysectomized group was partially hepatectomized (approximately 65 per cent of the liver removed) one week after hypophysectomy and sections of the removed left lateral lobe were fixed; these same animals were sacrificed 2 weeks later (at 42 days). At sacrifice the right lateral lobes from all the animals were fixed. All tissues were then sectioned at 15 μ and stained together. At least 1000 cells from each liver sample were scored. The distributions of cell types in hypophysectomized control animals sacrificed at 28 and 42 days were very similar and the results from these two groups have been combined in Table I, line 5 (the average percentage distributions of $2n$, $2n$ binucleate and $4n$ cells were 81.6, 14.4, 2.6 and 81.0, 13.1, 4.5 for livers from animals sacrificed at 28 and 42 days, respectively).

These data reveal several points of interest: (1) There are no significant differences between right and left lateral lobes with respect to the ploidy distribution patterns (lines 5 and 6). (2) Hypophysectomized and normal controls (lines 5 and 8) exhibit highly significant differences in the categories $2n$, $4n$ and $4n$ binucleate. In these two groups, the percentages of binucleate diploid cells present are not significantly different, probably reflecting similar levels on the ascending and descending parts of their frequency curve, shown in Fig. 1 of the preceding note [1]. (3) Finally, a comparison between lines 6 and 7 shows that a highly significant increase in polyploidy has taken place in the livers of hypophysectomized animals during regeneration. In fact, the polysomatic pattern in these animals is very similar to that of the normal controls listed in line 8.

In a discussion of his observations on polyploidy in human livers Swartz [7], following a suggestion of Leuchtenberger *et al.* [5], mentioned the possibility that growth hormone may be specifically responsible for polyploidization, but not for the general growth of the liver. The present results lead rather to the opposite conclusion since they show unequivocally that neither growth hormone nor any other pituitary hormone is indispensable for the process of polyploidization, *per se*. In intact animals growth hormone may well play a role in normal liver growth and concomitant development of polysomaty.

The mechanism responsible for polyploidy, and its control have not been definitively disclosed by any of these experiments. Nuclear fusion or endomitosis or both may be involved, while selective proliferation of higher polyploid cells is a less likely possibility. Unless it is controlled by a specific, still unknown factor, polyploidization may simply be a natural consequence of postnatal liver proliferation, perhaps resulting from a constant discrepancy between the rates of mitosis and cytokinesis.

Summary.—Although the progressive development of hepatic polysomaty is rested in the hypophysectomized rat, liver regeneration following partial hepatec-my in hypophysectomized rats is accompanied by an increase in the degree of poly-bidy. Thus, no pituitary hormone is indispensible for the development of poly-bidy.

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INCORPORATION OF ^{32}P INTO THE DNA OF RAT LYMPHOCYTES

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Received May 17, 1958

HE study of the life span of human lymphocytes by means of radioactive isotopic belling of the deoxyribonucleic acid (DNA) have been made in patients with chronic mphatic leukaemia, Hamilton [5] and Osgood, Tivey, Davison, Seaman and Li] and in two patients with normal white cell counts, Ottesen [8]. Since it is probable at the isotope is only incorporated into the DNA of these cells at the time of their nthesis, these observations indicate that the human lymphocyte may have a life an of several months and this may be prolonged in chronic lymphatic leukaemia. is isotopic technique has been used by Cossain and Kline [2] who found that in the bbit lymphocytes labelled with ^{32}P in the DNA could be recovered from the circula-n 15 days after giving the isotope by injection.

The thoracic duct lymph of rats contains an almost pure population of lympho-tes; this lymph can be obtained by an experimental fistula, Bollman, Cain and rindlay [1]. Hence it seemed that this preparation was most suitable for the study the survival of lymphocytes labelled with ^{32}P in the DNA. The initial experiment as made on female Wistar strain rats weighing 145–200 g which were given two ses of 0.5 μC ^{32}P/g body weight intramuscularly at six hour intervals. Pairs of rats were canulated at varying times after the injection of the isotope. The canulated rats were kept in restraining cages and given 5 per cent glucose in 0.9 per cent saline to drink to promote maximum lymph flow [6].

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[1] Clinical Research Fellow, Medical Research Council.

The rats were killed 36 hr. after canulation, the blood of the two animals was pooled, precipitated with 3 ml 30 per cent trichloroacetic acid (TCA) and the specific activity of the total phosphorus in the TCA supernatant fraction estimated. The lymphocytes were separated from the lymph by centrifugation and the specific activities of the DNAP estimated using the method of Deluca, Rossiter and Strickland [3]. The results of this initial experiment are shown in Table I. Since there was only a slight change of specific activity of the lymphocyte DNAP up to 28 days after the administration of the isotope, a second experiment was made in which the lymphocyte DNAP was compared to the thymus and lymph node DNAP specific activities over a period of 46 days.

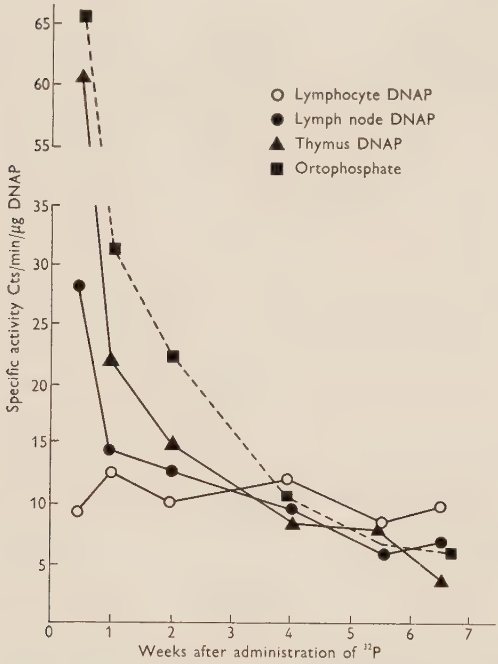

Fig. 1.—Persistence of ^{32}P labelling in the lymphocyte DNAP.

Male Wistar strain rats weighing 170–210 g were given the same dose of ^{32}P as in the previous experiment, canulated at intervals and killed 24 hr after canulation. The lymph nodes from the lumbar, mesenteric and cervical groups were mixed together and homogenized in 10 per cent TCA; the specific activities of the DNAP of the lymph node mixture, thymus and lymphocytes were determined as before. Fig. 1 shows the mean value of the specific activities of the DNAP of the lymphocytes, thymus and lymph nodes. The mean values are based on five observations at the 3rd and 46th days, the intermediate points on pairs of animals. The standard

TABLE I. *Specific activities (cts/min/μgP) of plasma and lymphocytes.*

(Corrected to day of injection of the ^{32}P.)

Day after isotope injection	TCA sol Plasma	Lymphocyte DNA
2	115	6.8
		5.3
7	15.6	7.6
		7.0
28	4.8	5.3
		6.0

TABLE II.

(All specific activities corrected to the day of injection of the ^{32}P.)

Days after injection of ^{32}P	Specific activity (cts/min/μg DMAP) Lymphocytes	Thymus	Lymph nodes
3	9.4 ± 0.28	56 ± 9.6	28 ± 5.5
7	12	24	12.2
	13.2	20.2	16
14	—	14.6	11.6
	10	15.6	15.5
28	14	7.5	8.6
	8.5	9.0	9.3
38	7.3	10.1	6.2
	9.5	5.3	7.0
46	9.9 ± 0.37	4.3 ± 0.67	7.0 ± 0.32

rors of the 3rd and 46th day observations and the individual intermediate values e shown in Table II. The estimates of the DNA specific activities were made on etween 5–88 μg of phosphorus. The experiment was ended after 46 days to avoid accuracies caused by low counts.

There was only a slight decline of the mean specific activity of the lymphocytes, though there was a considerable scatter of the values of individual samples. On the other hand the thymus and lymph nodes both show a far greater change of specific activity than the lymphocytes. The fall of specific activity of the thymus DNAP tends to run in parallel with the specific activity of the orthophosphate. It has been shown in another experiment that the intensity of labelling of the lymphocyte DNAP *in vivo* and *in vitro* is proportional to the orthophosphate ^{32}P concentration. The concentration of ^{32}P as orthophosphate in the blood of rats undergoes a rapid fall during the first few hours after its injection intramuscularly into the animal [4]. This phase of rapid decline leads into a slower exponential fall of ^{32}P concentration lasting for many weeks. Lymphocytes that are synthesised shortly after the injection of the ^{32}P will have a much higher specific activity in their DNAP than lymphocytes synthesised when the orthophosphate pool has a lower specific activity. Since there was only a slight fall of specific activity of the lymphocyte DNAP from the 2nd to the 28th day in the first experiment and from the first to the sixth week after giving the ^{32}P in the second experiment, it is probable that a large proportion of the lymphocytes that incorporated ^{32}P into their DNAP shortly after the isotope was injected are still alive six weeks later.

Whilst these experiments have not determined the life span of the rat lymphocyte, they do provide evidence from another animal that the lymphocyte has a long life span. The β-radiation from the ^{32}P may have caused a significant effect upon the lymphocyte life span in this experiment and further experiments are in progress to check this point.

I wish to thank Dr. J. L. Gowans and Dr. L. A. Stocken for their advice and encouragement with these experiments.

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N THE REFRACTIVE INDEX OF HISTOLOGICAL TISSUE SECTIONS IN VISIBLE AND IN ULTRAVIOLET LIGHT

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Received March 27, 1958

the course of our work on the distribution of ultraviolet absorbing substances in in tissue sections it was found desirable to use methacrylate sections, from which e embedding medium has not been removed. In order to be able to assess the use- lness of media other than those hitherto customary in ultraviolet microscopy g. pure glycerol [1], glycerol saturated with chloralhydrate or zinc chloride [3]), e have measured the refractive indices at various wavelengths in both the visible nd ultraviolet ranges of fixed tissue sections and of a number of potential imbibi- on media including polymerized butylmethacrylate (with an admixture of 5 per nt methylmethacrylate).

Method.—The measurement of the refractive indices of the tissue has been per- rmed by first determining the dispersion curves of a number of imbibition fluids nd next by finding the best match at a few selected wavelengths of these fluids with e tissue section.

The refractive indices of the liquids used have been measured in a simple refracto- eter (see Fig. 1), consisting of a quartz slide (S) and two optically polished quartz ps (C_1 and C_2). The coverslips are attached to a brass ring so as to enclose between em and the slide a shallow cell consisting of two prismatic spaces with a common fracting rib. The liquid to be measured is retained in these spaces by capillary rce. This cell, when introduced in the light path of a mirror yielding an image of the it slit of a monochromator, causes the slit image to be doubled. From the geometry the apparatus and the mutual distance of the two slit images, the refractive index the liquid is computed. With a refracting angle of the prismatic spaces of 5°, the spersion curve (from 578 mμ to 248 mμ inclusive) of distilled water could thus be easured to an accuracy of one unit of the third decimal place. The dispersion curve polymerized methacrylate has been determined in the same refractometer. To is end, a drop of prepolymerized methacrylate was allowed to polymerize *in situ.* urthermore, measurements have been made on a thin wedge of methacrylate em- dded in water and in glycerol.

The matching of the refractive indices of the medium and the tissue section has en checked by microphotography under conditions of dark field illumination. A flecting objective[1] has been used, in connection with a reflecting condenser, the aximum aperture of which was just below the aperture of the central stop in the jective. Though under these conditions the dark field picture is not as easily interpreted as when the condenser aperture exceeds that of the objective, the main features of the section are easily recognizable and any slight mismatch in the refractive indices of fluid and section shows up very brightly. Apart from the brightness of the dark ground pictures, the frequently striking differences between the pictures obtained with too low or too high refractive indices respectively of the immersion medium are helpful in finding the correct match.

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[1] This objective (num. apert. = 0.85) was built a few years ago by the optical firm "Oude elft", Delft, The Netherlands. It is a quartz objective with spherical mirrors. The linear ob- uring ratio is 22 per cent.

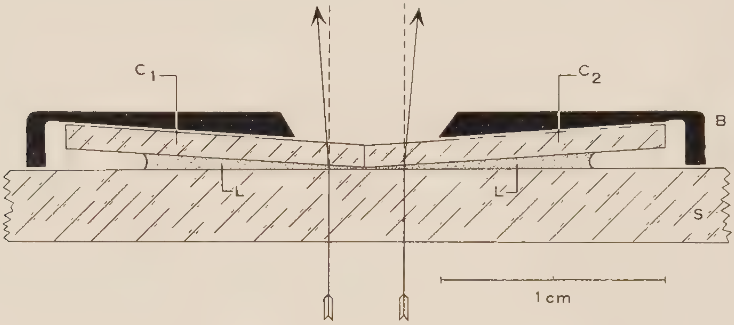


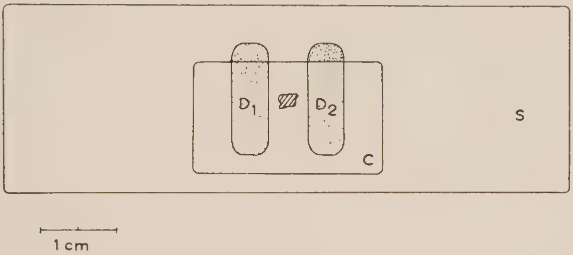
Fig. 1.—Diagram of the refractometer cell used for determining the refractive indices of the imbibition fluids. *S*, quartz slide; C_1 and C_2, quartz coverslips; *B*, brass ring; *L*, sample to be measured. The light path has been indicated by arrows.

Both the refractometer and the microscope derived their illumination from a mercury arc, the following spectral lines of which were isolated by a monochromator: 578, 546, 436, 405, 366, 334, 312, 302, 289, 280 and 265 mμ.

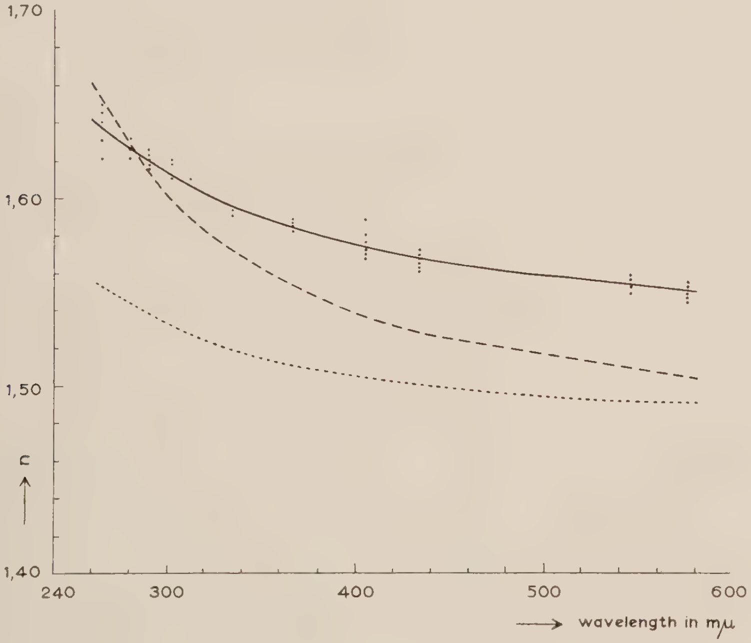
In order to facilitate the renewal of the imbibition medium of an object during the observation, a simple perfusion chamber of minimal depth has been constructed (see Fig. 2). In a quartz slide *S*, which in our microscope is to be used in a vertical plane, two depressions (D_1 and D_2) have been ground. The coverslip *C*, onto which the section is stuck, is firmly clamped onto the slide, so as to leave the upper parts of the depressions uncovered. Through two finely drawn out glass tubes dipping into these depressions, a constant flow of the liquid across the section can be effected. Though the distance between the slide and the coverslip does not exceed the thickness of the section, the complete renewal of the fluid within a small section is a matter of minutes.

Results.—The results of a number of measurements on rat liver sections fixed in either Bouin's, Zenker's or Carnoy's fluid, embedded and cut in paraffin and deparaffinized in xylene, along with the dispersion curves of a few media, are presented in the graph of Fig. 3. The data on the tissue have been obtained with mixtures of benzene and cyclohexane at the wavelengths 265 and 280 mμ. For the range 289 mμ to 334 mμ inclusive, mixtures of methyl-benzoate and decaline were found to be suitable, whereas at longer wavelengths including the visible range media containing α-chloronaphthalene and butylcarbitol were used. Within single sections different parts (notably erythrocytes, nucleoli) presented slightly different matches. Similarly, the dispersion curves of differently fixed tissues and of sections with different histoes did not coincide exactly. We did not, however, further investigate these differences, which did not exceed a few units of the second decimal place, as they seem be unimportant as causes of serious diffraction effects.

Discussion.—Whereas data as to the refractive index of tissue sections in the isible light are fairly numerous (e.g. [2, 4, 6]) and well within the range of our reults, we are aware of only one report [5] on the determination of the ultraviolet



g. 2.—Quartz perfusion chamber for thin sections. *S*, quartz slide; D_1 and D_2, depressions, ound in *S*; *C*, coverslip onto which the section is stuck. Clamps and glass tubes omitted.



g. 3.—Dispersion curves of histological sections (———), benzene (– – – –) and of polyerized butylmethacrylate, mixed with 5 per cent methylmethacrylate (· · · · · ·). The latter rve almost coincides with that of a concentrated solution of chloralhydrate in glycerol (not awn). Actual measurements on tissue sections are represented by dots.

refractive index of fixed tissue. Our results confirm the prediction of Rudkin and Corlette to the effect that the ultraviolet refractive index of fixed cell material should be somewhat higher than the computed $n = 1.60$ of their most refractive glycerol-zinc-chloride mixture.

Whether polymerized butylmethacrylate can be considered a suitable medium for ultraviolet photography depends on the nature of the investigation. Judging by its dispersion curve it is equivalent to glycerol-chloralhydrate mixtures. If, however, there is no objection to the methacrylate being removed from the section, the use of a medium with a higher refractive index, e.g. benzene, which in thin layers is sufficiently transparent down to 265 mμ, seems to be safer. A number of other fluids has been tested, but we have been unable to find a medium with approximately the same dispersion curve as fixed tissue sections.

The untiring and skilful assistance rendered by Miss C. W. Leeflang is gratefully acknowledged.

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A RAPID METHOD FOR THE QUALITATIVE ANALYSIS OF FREE AMINO ACIDS IN ANIMAL TISSUES BY PAPER CHROMATOGRAPHY

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Received June 3, 1958

Ever since the first application of chromatographic methods on filter paper by Consden, Gordon and Martin [1] in 1944, tremendous progress has been made in the use of the equipment and the overall improvement of the method by modifying the moving phase and the developing technique, but comparatively less attention has, however, been paid to the preparation of the material for analysis. In the case of the free amino acids in animal tissues it has been customary to prepare extracts of tissues by deproteinising with alcohol, trichloracetic acid or tungstic acid and subject them to analysis after adjusting the pH. In this paper is outlined a rapid procedure which does not require extraction and which makes possible the analysis of free amino acids in small quantities of animal tissues by paper chromatography. A similar method as been described by Herrmann for the study of cell constituents in soft plant ma- rial [5].

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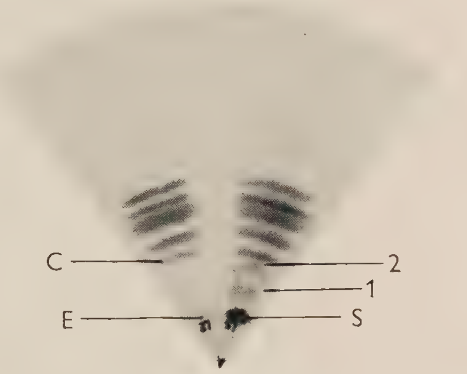
[1] This author is indebted to the Government of India for the award of a Senior Research Scholarship which made this work possible.

The general procedure adopted was by no means different from those followed by rlier workers. The circular paper chromatographic method described by Giri and ao [4] and Giri *et al.* [3] was employed in this study. Whatman No. 1 filter paper 18 inches diameter was used. Chromatograms were run twice with *N*-butanol-etic acid-water (40:10:50), dried at room temperature, sprayed with 0.2 per cent nhydrin in 95 per cent acetone and heated for 15 minutes in an air oven at 65°C develop colour.



g. 1.—Chromatogram of an alcohol extract and section of liver of rat showing separation of e amino acids. *E*, extract, *S*, section, *C*, cystin, and 2, extra bands.

The materials used were tissues such as muscle, liver and kidney of frog, pigeon d rat. Fresh sections of frozen tissues were cut and applied directly on the paper stead of the extract. Sections were cut according to the method described by George d Scaria [2]. Tissues cut from animals soon after they were decapitated and bled death were placed over ice in the deep freeze of a refrigerator. On freezing, which kes only a few minutes, they were trimmed to shape and very thin sections were t with the aid of a cold, sharp razor blade. After removing the water from the blade means of filter paper, the section was carefully transferred to the Whatman paper pressing it against the paper and slowly drawing out the blade sidewise. This sures easy transference of the entire section from the blade to the paper and if cessary one finger of the other hand which is free may be applied to the lower sur- ce of the paper while transferring the section. On the other hand if the tissues be- me very hard sections did not adhere to the blade and fell off on ice, they could transferred to the paper with the aid of a cold pointed forceps. The sections were ced along the circumference of a circle of two inches in diameter at regular dis- nces alternating with spots of alcohol extracts of the same material, acid hydro- ate of casein and standard mixture of known amino acids for comparison. The romatograms were then run as usual.

Fig. 1 presents in photograph a chromatogram of a section as well as an extract the liver of rat prepared in the manner described above. It is seen clearly that the tion gives all the bands characteristic of the extract. This was found to be true th all the tissues tried. Chromatograms of sections cut on a freezing microtome also ve the same results. The residual section left on the paper was subjected to extraction and the extract tested for amino acids. The negative results obtained indicated that no free amino acids were left in the residue. In the case of some tissues like liver, kidney and the pigeon breast muscle which are metabolically very active, two additional bands which are light bluish purple in colour appear below cystin (Fig. 1) in chromatograms of sections. These bands do not correspond to those of any of the common amino acids. Evidently these bands are some rare amino acids, peptides or simple proteins. Saifer and Oreskes [6] have listed a number of substances which have R_f values lower than cystin when butanol-acetic acid-water (40:10:50) is used as the solvent and ninhydrin as the developing reagent. The exact chemical nature of these two bands is under investigation.

The advantages of this method are that it is time-saving and requires only very small quantity of tissue. Section cutting does not require a microtome and can be done in any laboratory having a refrigerator. This method may be profitably employed for the analysis of free amino acids in isolated parts of embryos and organisms like protozoans and other small animals. It might also prove useful in the study of organic acids and other substances in biological material. It should be of interest to mention here that the section method can be used with equal advantage for the quantitative estimation of free amino acids in tissues (George and Pishawikar, unpublished). In this case the exact quantitity of the tissue spotted has be determined and which could be done if the sections are cut on a freezing microtome.

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A REPORT OF SEX CHROMATIN IN HUMAN TUMOR TISSUE CULTURE

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Received June 15, 1958

The sex chromatin of somatic interphase nuclei was first reported by Barr *et al.* [1] as being a common feature of female cat neurones though relatively rare in the male. Since then this deeply basophilic, Feulgen positive [5] intranuclear mass has been used to identify the genetic sex of individuals of various species. Some of the

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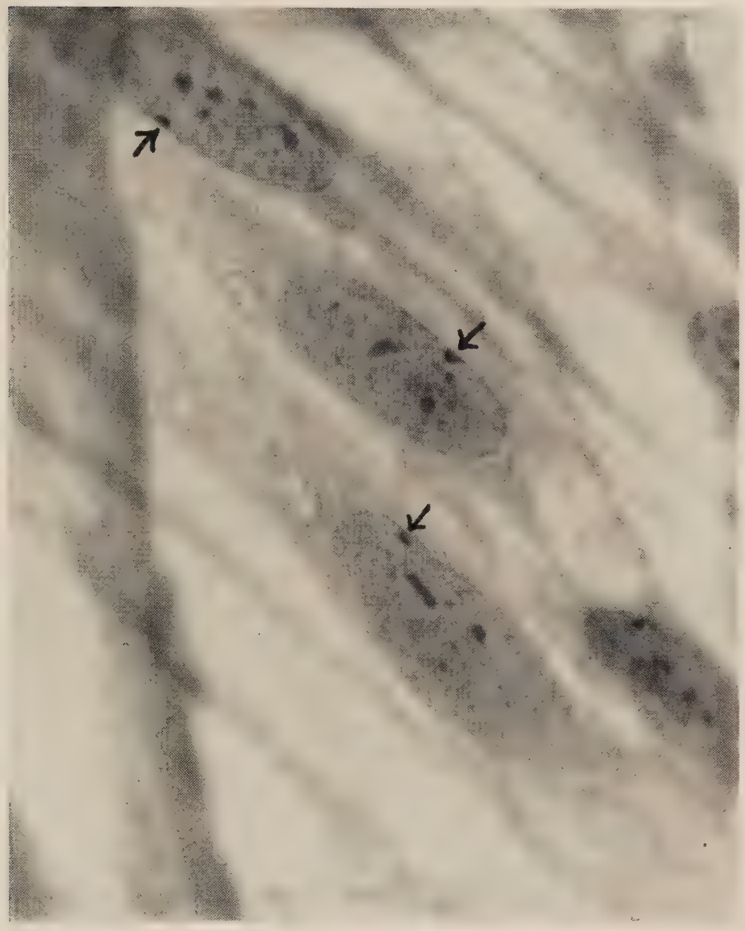
[1] The authors wish to thank Mr. Robert Wood for the photomicrography and Mrs. E. Manoogian for assistance in the preparation of the manuscript.



ig. 1.—Hematoxylin-eosin stained mammary tumour tissue culture. Arrows indicate the sex romatin mass adjacent to the inner side of the nuclear membrane. The middle sex chromatin of the wedge-shaped type in contrast to the other two which are more planoconvex. All three x chromatin are the most deeply stained cellular components. × 1620.

urces for sex chromatin studies have been fixed and stained sections of normal nd neoplastic tissue [7], unfixed smears from the mucosa [10], and fetal cells from uman amniotic fluid [9]. Regardless of source, the shape, size, staining properties, nd position within the nucleus have been remarkably similar. No published reports ave been found of a similar intranuclear mass in either primary or established tissue ultures and, in this laboratory, examinations of HeLa [4] and rabbit kidney "Drew ine" [2] cultures which are of known female origin have never disclosed any sem- lance of sex chromatin. The failure to demonstrate sex chromatin in long established ssue cultures might be due to its loss or masking following extensive *in vitro* propa- ation. We have found evidence for sex chromatin in hematoxylin-eosin and Feulgen

[3] stained cells of a recently established cell line of human mammary tumor origin obtained from Dr. V. J. Cabasso of this laboratory. The deeply basophilic wedge-shaped or planoconvex mass (Fig. 1, arrows) which is usually found adjacent to the nuclear membrane is strongly Feulgen positive. It also resembles the sex chromatin of somatic tissue in size and frequency of appearance. Measurements were made of the first 20 sex chromatin found adjacent to the nuclear membrane in three randomly selected cultures using a 2 mm Leitz aprochromat and a Bausch & Lomb Filar micrometer. At least one full sweep of the width of the culture slip was required to obtain 20 nuclei with this specific chromatin mass. The frequency of sex chromatin was obtained by counting 200 cells from each of the three cultures. Both sex chromatin adjacent to or free of the nuclear membrane (approx. 25 per cent) were counted. Table I summarizes the similarity between somatic tissue sex chromatin [1, 6, 8] and that found in this tissue culture line. Since these cells have been growing for only ten weeks (nine transfers) it is possible that in time their sex chromatin will be no longer demonstrable. Therefore, samples of each transfer are being taken to observe any changes in or the disappearance of the sex chromatin. These changes might serve as genetic indicators of cellular variations in morphology, metabolism, and virus susceptibility which are known to occur in cells following prolonged sub-culturing [6].

TABLE I. *Comparison of sex chromatin.*

	Somatic tissue		Tissue culture	
	Human & primates—all types		Mammary tumor origin	
	Average	Range	Average	Range
1. Size (microns)	0.8 × 1.2	0.6 × 1.1 0.9 × 1.3	0.9 × 1.6	0.7 × 1.3 1.2 × 1.6
2. % Cells with sex chromatin (in female tissue)	73.6	(66–80)	62.5	(60–64)
3. Feulgen positive	yes		yes	
4. Deeper basophilia than rest of chromatin	yes		yes	
5. Usually located on inner surface of nuclear membrane	yes (approx. 3/4)		yes (approx. 3/4)	
6. Usually wedge-shaped or planoconvex	yes		yes	

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THE FINE STRUCTURE OF CARDIAC MUSCLE CELLS OF NEWBORN AND SUCKLING MICE[1]

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Received June 17, 1958

HE fine structure of normal, adult heart has come under considerable study re- ntly, with particular emphasis on its cellular nature, and the meaning of the inter- lated discs [3, 6, 7, 8]. Investigations of development of heart muscle include myo- bril development in the chick embryo [2] and intercalated disc formation in the rabbit]. We have begun a study of the changes that occur in mouse cardiac muscle cells ith age, starting with the earliest embryonic stages and proceeding through to the dest available adult. Of major interest is the development immediately preceding nd following birth and the maturation through the weaning stage, particularly nce it has been found that the newborn and three-day-old animal still possess an nmature cardiac muscle structure.

The ventricular apices of hearts from mice less than twelve hours old and also om mice three days old were fixed and embedded by standard methods. Sections ere obtained using diamond knives in Porter-Blum microtomes and examined in e Siemens Elmiskop I at initial magnification of 2600 × to 10,000 ×.

The cardiac muscle cells of the newborn mouse (Fig. 1) are characterized by large umbers of mitochondria, a sparseness of myfibrils, and by many Palade granules scattered rosettes and along the often widely separated membranes of the endo- lasmic reticulum. The Golgi apparatus is prominent and the nuclear envelope com- lete. The cytoplasmic matrix contains fine, unoriented filaments. The cell border ows a total cytolemma on the interstitial side, but plasma membrane only where eighboring cardiac cells adjoin. Well formed intercalated discs appear in those re- ions where the longitudinal fibril pattern is interrupted by cell membranes. The num- ers of mitochondria are significantly greater than is observed either in early embryos r in late adult stages. They are generally nebulous in shape; showing little or no referred orientation with respect to the fibrils, and contain many cristae of unusual inness and which also show a lack of alignment. The myofibrils appear to be incom- lete, lack the definitive adult parallel arrangement, and at their termini are closely ssociated with the fine cytoplasmic filaments, granules and mitochondria, suggesting at all three cytoplasmic components enter into fibril formation. The membranes f the endoplasmic reticulum may be closely apposed or widely separated, forming cunae. In most instances they are associated with Palade granules along their cyto- lasmic border.

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[1] This study was aided, in part, by a grant (H-3493) from the National Heart Institute, of the epartment of Health, Education and Welfare.

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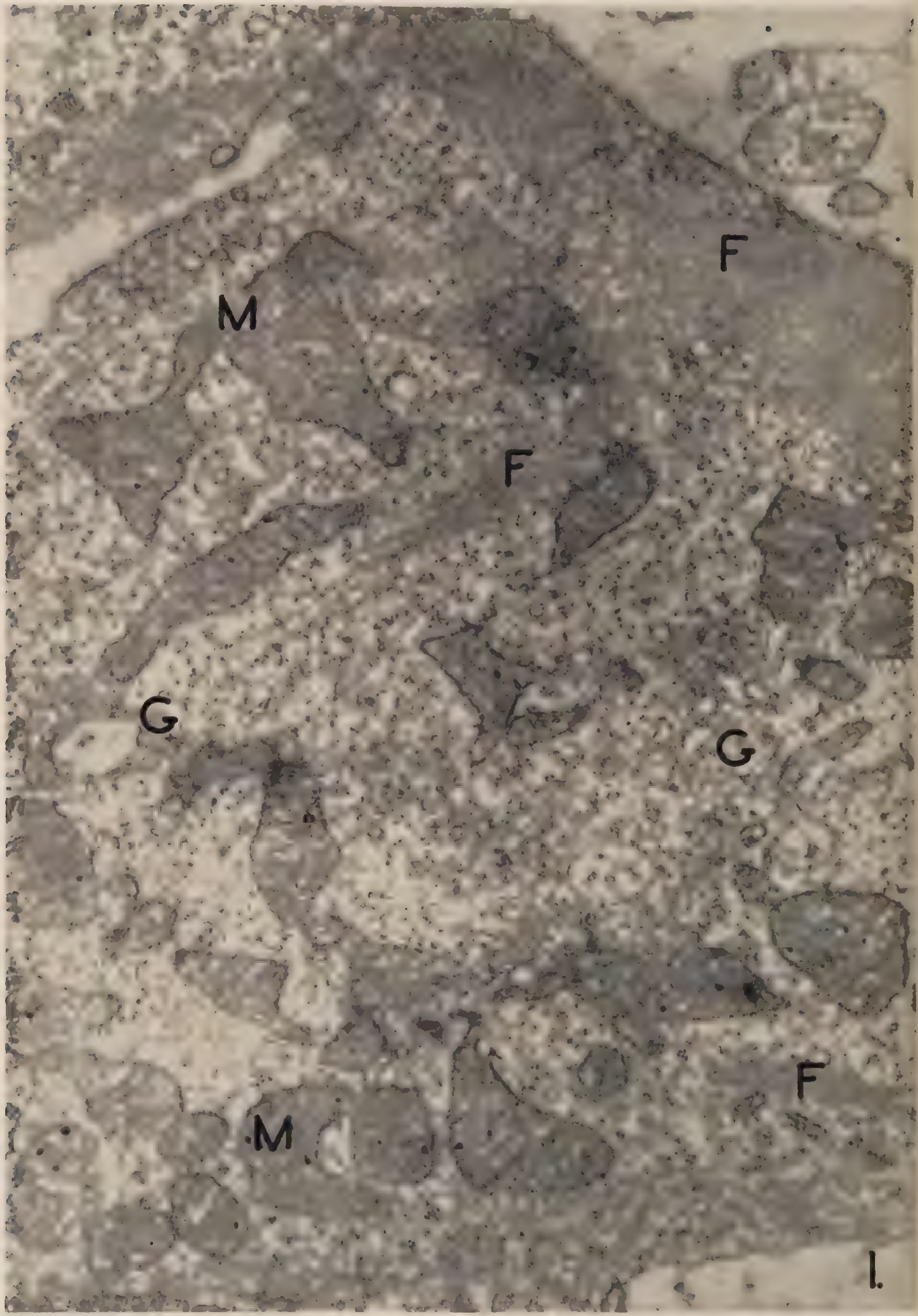
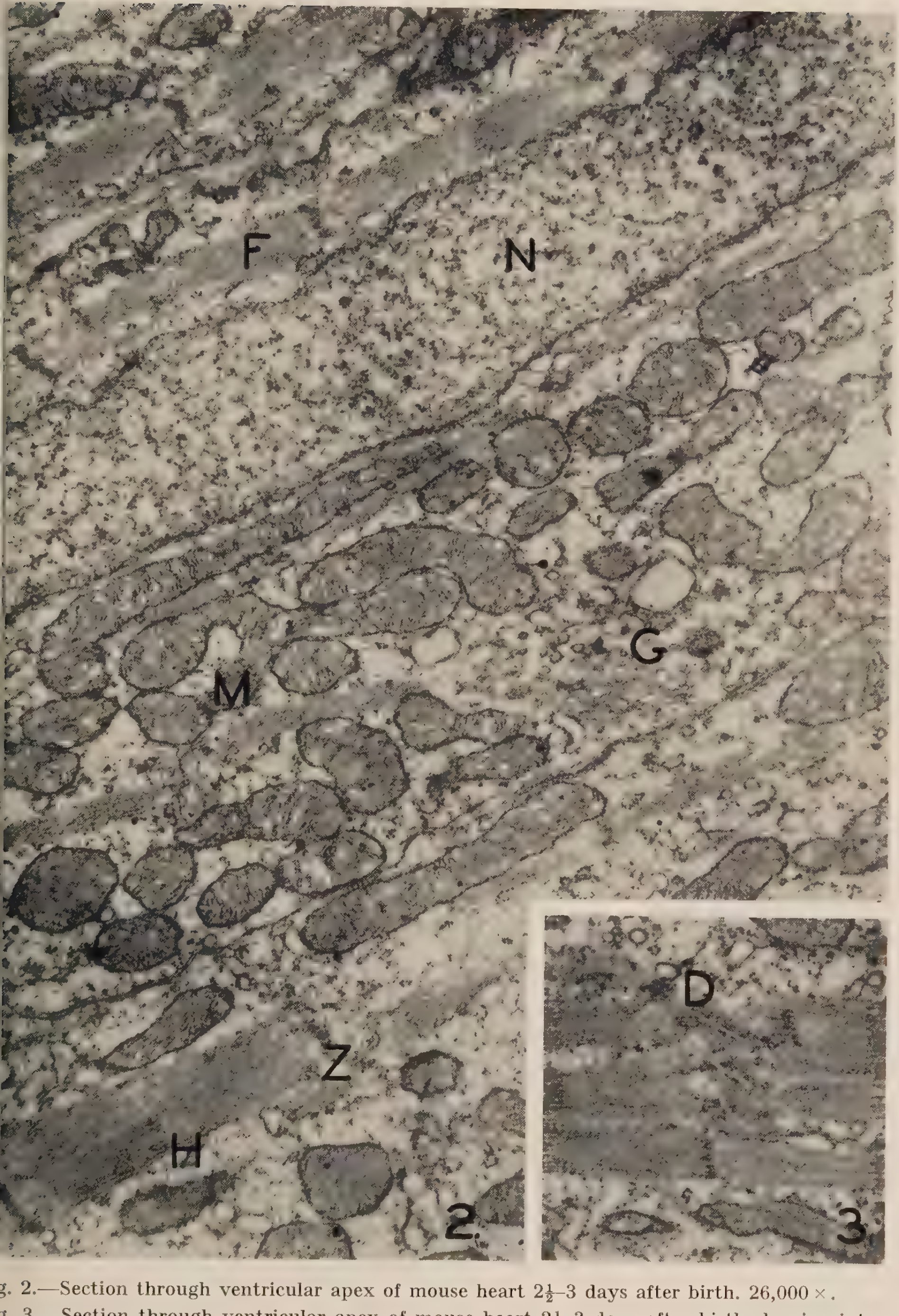


Fig. 1.—Section through ventricular apex of mouse heart less than 12 hours after birth. 25,000 ×.



g. 2.—Section through ventricular apex of mouse heart 2½–3 days after birth. 26,000 ×.

g. 3.—Section through ventricular apex of mouse heart 2½–3 days after birth showing interated disc. 30,000 ×.

y.—*D*, intercalated disc; *F*, fibrils; *G*, Golgi apparatus; *H*, H band; *M*, Mitochondria; *N*, Nucleus; Z band.

The heart muscle fibers of the three-day-old mouse (Fig. 2) appear more like adult conducting system [1, 5] than definitive, adult, heart muscle. As in the newborn, the cell borders and discs are well formed, and the nuclei possess envelope and matrix. The cytoplasmic matrix still consists, however, of numerous fine filaments and Palade granules. Significantly, the mitochondria are now more regular in outline and the tightly packed cristae mitochondriales show some orientation and are noticeably thicker. Also, the mitochondria themselves show a preferential alignment parallel to the myofibrils. The latter are increased in number, but close to the cell border and seldom number more than two per fibril bundle in the plane of sectioning. The fibrils are still intimately associated with granules, fine filaments and mitochondria at their ill-defined ends. However, the sarcomeric pattern of regions and cross bands has now become apparent, whereas the H band, and the demarcation between A and I regions were not easily distinguishable in the newborn.

Two important points have become apparent in the present study. Firstly, that large numbers of mitochondria, filled with cristae, are present in the heart muscle fibers immediately after birth, indicating a high rate of oxidative metabolism. Secondly, that myofibril formation is still at an early stage in the strongly-beating heart of the three-day-old mouse, suggesting that the definitive adult structure must be attained slowly during the suckling period and that fibrillar apposition takes place by training.

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THE LABELLING OF MOUSE SPERMATOZOA BY ADENINE-8-^{14}C AND THYMIDINE-^{3}H

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Received June 18, 1958

The labelling of mouse spermatozoa has been achieved by intraperitoneal injection of about 1 μc adenine-8-^{14}C per gram body weight [6]. This labelling permits the sampling of male pronuclei [2] and abnormal sperm derivatives [4]. It also permits alysis of the maturation of spermatogenic cells during normal or interfered sperma-genesis [3, 7] and of percentages of spermatozoa in mixtures [7]. The possibility at the technique could be made more economical by injection of adenine into testes of tritiated thymidine has been pointed out [7], and experiments along these lines e reported here.

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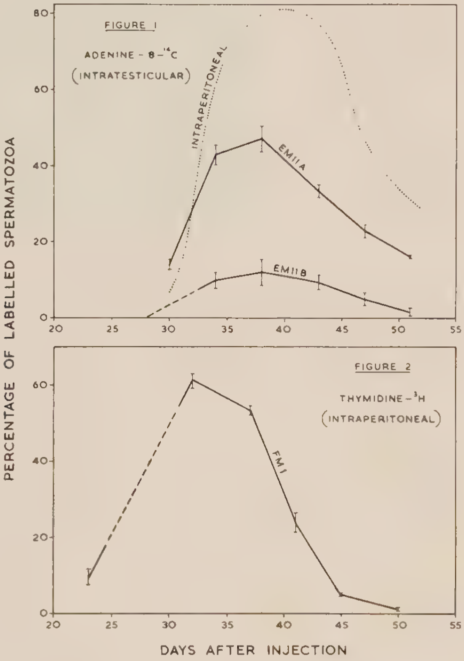
[1] Research worker of the British Empire Cancer Campaign.



gs. 1 and 2.—The percentage of labelled sper-atozoa ejaculated at various intervals after ection of tracers.
ferences to Figs. 1 and 2. One standard error × S. E.) plotted above and below means. The tted graph in Fig. 1 is the estimated average several males injected with about one μc enine/gram body weight and 50 days exposure films [7].

Material and technique.—Spermatozoa of outbred mature male mice (average ight 32 g) injected with tracers were collected from uteri of females mated to ese males, either spontaneously or after superovulation. Smears were prepared r autoradiography with Kodak AR.10 film and exposed for 50 days. Percentages labelled spermatozoa were determined by successive counts on two groups of fifty ermatozoa of normal appearance in each of two replicate preparations. A X^2 test owed no heterogeneity between replicate counts and indicated a tendency to ho-ogenize successive scores, which should reduce the standard errors probably without ich affecting the graphs otherwise. Spermatozoa producing either definite or weak toradiographs, mostly due to weak labelling, were scored as positively labelled.

Intratesticular injections of adenine.—Two males were anaesthetized and injected to the testes with the same fresh solution of adenine-8-^{14}C (Radiochemical Centre, gland; 9.6 mc/m*M*), using a needle No. 27. Male EM11A was injected through the otum (ca 4.6 μc in 0.02 c.c. between both testes) and male EM11B through two otal incisions (ca 6.8 μc). Both males were paired with females at about 7, 14 and days post-injection (p.i.) and mated normally.

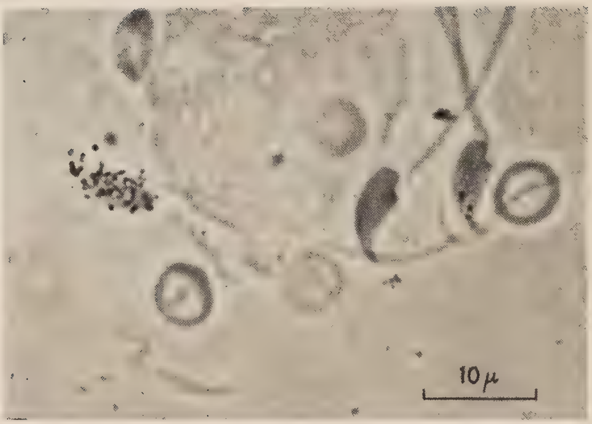


Fig. 3.—Autoradiographs on spermatozoa after injection of thymidine. Three sperm-heads: one weakly (right) and one strongly labelled (left), another not labelled (center). Also red blood and vaginal epithelium cells. Phase contrast.

Samples studied with autoradiography (Fig. 1) show that the ejaculation of labelled spermatozoa by these two males follows the same pattern as when adenine is given intraperitoneally. Spermatozoa can therefore be labelled with quantities of adenine several time less than those given intraperitoneally but, as suggested by Fig. 1, the strength of labelling may be more variable between males.

Intraperitoneal injection of thymidine.—One male was injected with 33 μc thymidine-^{3}H (Schwarz, U.S.A.; 208 mc/mM), labelled in the thymine. Spermatozoa were not radioactive at 2.5, 5.5 and 16 days p.i. Later samples (Fig. 2) show that the ejaculation of labelled spermatozoa occurred earlier than in males given adenine: the whole graph for this male is displaced towards shorter intervals than the average graph in Fig. 1, or than any other single male [7]. As to the ejaculation of thymidine-labelled spermatozoa at 23 days p.i., it should be noted that only one (EM2D:3) out of many males has ejaculated adenine-labelled spermatozoa before 30 days. The tracer was confined to sperm-heads and not observed in seminal plasma.

The pattern of labelling with thymidine indicates that its tritium does not interchange with hydrogen, for if it did interchange then labelled spermatozoa would appear in ejaculates much earlier (first days p.i.) and also later than with adenine or the seminal plasma be labelled. The labelling before 30 days, if confirmed, indicates a greater turn-over of thymine (cf. [8]) than of adenine after DNA has been synthesized during preleptolene. The earlier disappearance of thymidine-labelled spermatozoa from the ejaculates is likely due to lack of a reservoir for thymine similar to that for adenine proposed by Pelc [5] in RNA.

Staining shows that most of the head, except the distal acrosome, is occupied by the nucleus. Tritium is homogeneously distributed and more abundant in the main body of the nucleus and decreases towards its most anterior and posterior parts (Fig. 3), indicating corresponding amounts of DNA. Photographic grains associated with the most strongly labelled heads, usually within 0.5 μ outside their boundaries,

ere counted as described before [7]. Counts were similar between replicate prepara-
ons and the mean net counts were 49.8 ± 2.5 at 32 days and 41.4 ± 1.7 at 37 days
i. (less than 0.6 background grains). As with adenine, the average maximum tracer
ntent is then greatest at the peak of labelling [7]. Some saturation of the film is
pected at these grain densities as tritium-electrons rarely traverse the basalmost
ain layer. Self-absorption is also expected as the average electron will traverse
ss than one micron in matter of unit density and therefore even less in the denser
erm-heads, slightly less than one micron thick. To conclude, it is feasible to label
ermatozoa with thymidine at considerably less cost than with adenine, but autora-
ography of pronuclei derived from them may be impaired.

Comparison of the labelling attained with the two tracers.—From the counts for male
M1 at 32 days p.i. it is calculated that sperm-heads with maximum labelling had
corporated $> 3 \times 10^{-15}$ m*M* tritiated thymidine (geometry: < 50 per cent; assumed
eld: one grain/electron [see above]). One sperm-head contains about 3.0×10^{-12} g
NA and 2.82×10^{-12} m*M* thymidine (1).[1] The labelling was therefore $> 1{:}940$.

From the maximum counts for male DMI0A ([7], Fig. 3) at the peak of labelling
ter injection of adenine-8-^{14}C, i.e. 37 days, it is calculated that sperm-heads had
corporated $> 10^{-13}$ m*M* ^{14}C-8 atoms (yield: 2 grains/electron). One sperm-head con-
ins 2.88 and 2.01×10^{-12} m*M* adenine and guanine [1]. The labelling was therefore
1:28.8 if the tracer was only incorporated into adenine, or $> 1{:}48.9$ if it entered
qually into adenine or guanine.

The author wishes to thank Professor C. H. Waddington, F. R. S. for his interest,
r. M. Latyszewski for the injections into testes, and Dr. M. Mitchison for the gift
the thymidine.

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[1] Calculated with the average molar proportion of bases given in table 9 (1) and the resulting
.02 per cent DNA-P.

A METABOLIC ISOTOPE TRANSFER FROM SOLUBLE POLYNUCLEOTIDES TO MICROSOMAL NUCLEOPROTEIN IN A CELL-FREE RAT LIVER SYSTEM

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Received June 23, 1958

In cell-free liver systems labeled amino acids are incorporated into microsomal proteins by way of a two-step reaction mechanism [7, 10, 8, 2]. By the first step, in which ATP is required as a source of energy, the amino acid becomes attached to a soluble polynucleotide ("S-RNA"). A subsequent transfer of this bound amino acid to a reactive microsomal site apparently constitutes the second reaction step. A transfer of this kind can actually be demonstrated in microsomal systems under proper conditions.

In view of the close connections between protein and nucleic acid metabolisms [6, 1, 14, 15] we were interested to know, whether or not components of the soluble RNA-fraction were transferred to microsomal RNA under the conditions of amino acid incorporation into microsomal proteins. It is well known that in isotope experiments *in vivo* the soluble RNA-fraction becomes labeled more rapidly than the microsomal RNA [12, 11, 4]. This may possibly indicate that a transfer of soluble RNA (or RNA-components) to microsomal nucleoprotein actually takes place in living cells.

Experimental.—By 2–3 subcutaneous injections ^{14}C-adenine (0.1 mc) or ^{32}P-phosphate (1–5 mc) were administred to male albino rats, weighing about 100 g. (Hepatectomized rats were used in some experiments.) About 15–20 hours after the last injection the liver was taken out and homogenized in a solution of 0.025 *M* KCl, 0.010 *M* $MgCl_2$ and 0.035 *M* tris-(hydroxymethyl)-aminomethane, pH 7.8. A soluble fraction was prepared in the usual way. It was dialyzed against large volumes of ice-cold homogenization medium for about 20 hours, with frequent changes (Visking dialysis tubing, 10 mm). The diffusion of low molecular, labeled compounds through the membrane was followed by radioactivity measurements. After dialysis the solution was centrifuged for 2 ½ hours at 105,000 × g (0°C). In some experiments ATP and an amino acid mixture were added to the homogenate before fractionation, with the intention to facilitate the release of protein-bound nucleotides [5].

In the interval, microsomes were prepared from non-radioactive regenerating rat livers. The homogenization medium (2.5 volumes) had the same ionic composition as in the previous case, but contained in addition 0.15 *M* sucrose. The homogenate was centrifuged at 14,000 × g for 7 minutes. The supernatant was carefully added as a separate layer at the top of 30 ml centrifuge tubes containing the same medium but with 0.30 *M* sucrose. The tubes were centrifuged for 60 minutes at 79,000 × g. The sedimented microsomes were resuspended in the sucrose-free medium.

The incubation system contained 2.0 ml of labeled, dialyzed supernatant and 1

TABLE I. *Requirements for isotope transfer from soluble polynucleotides to microsomal nucleoprotein by a rat liver system.*

The prelabelled soluble fraction had been dialyzed for 20 hours. The microsomes had been epared from non-labeled, regenerating liver. The tubes (final volumes 5.0 ml) were incubated 8 minutes at 35°C, except the control tubes, which were left in the ice-bath. PEP = phos oenolpyruvate (30 μM); ATP, GTP, UTP. CTP = adenosine, guanosine, uridine and cytidine triphosphate (3 μM).

	Supernatant prelabeled with ^{32}P-phosphate C.p.m. per 100 μg phosphate		
	Cytidylic acid	Uridylic acid	^{14}C-adenine C.p.m. per mg RNA
crosomes, labeled supernatant			21, 11
ıs ATP	230	260	
PEP	130	220	
PEP, ATP	870	800	51, 35
PEP, ATP, amino acids	750	830	52
PEP, ATP, GTP, UTP	960	915	
PEP. ATP, GTP, UTP, amino acids	970	930	
PEP, ATP, GTP, UTP, CTP			43
PEP, ATP, GTP, UTP, CTP, amino acids			58, 46
bt in ice-bath during incubation	200	360	17, 14

of the microsomal suspension (corresponding to about 2 g of fresh liver). The ects of the following additions were tried in varied combinations: 3 μM of ATP, P, CTP and UTP, 30 μM of phosphoenolpyruvate, and 9 μM of each of the fol- ving amino acids (L-forms): alanine, arginine, glycine, histidine, isoleucine, leucine, ine, phenylalanine, serine, threonine, tryptophane and valine. The final volume s usually 5.0 ml.

The mixtures were incubated at 35°C for 8 minutes. One tube was left in the ice- th during the incubation period. After the incubation the suspensions were care- ly layered at the top of 30 ml centrifuge tubes containing 0.3 per cent deoxycholate 13] in 0.15 M sucrose. The tubes were centrifuged for about 3 hours at 79,000 × g. ter the centrifugation only the top layer was colored. An almost colorless pellet d accumulated at the bottom, separated from the colored layer by a broad, per- tly colorless zone. The pellets were carefully drained and rinsed with medium, d the tubes were dried with filter-paper. The sediments were treated with cold chloroacetic acid (TCA), lipid solvents and hot TCA. The extracts with hot TCA re treated with ether in extractors. The absorption curves were determined and d as a measure of the RNA-contents. In the ^{32}P-experiments the RNA-nucleotides re further hydrolyzed with 0.5 M KOH and separated by Dowex 2 columns [3].

In both kinds of experiments the specific activities of the microsomal RNA-prepa- ions were constantly higher in the incubated, complete system than in the control tubes kept in the ice-bath (Table I). When ATP was omitted from the system, the incorporation was reduced to about the same level as in these controls. A significant reduction was also observed in the absence of phosphoenolpyruvate.

It seemed possible that the incorporation might be due to labeled nucleoside triphosphates, not entirely removed from the soluble fraction by the dialysis, or produced during the incubation by pyrophosphorolysis [15]. In order to test this possibility a mixture of ribonucleoside triphosphates (ATP, GTP, CTP, UTP) was added to the system for isotope dilution. The incorporation was not reduced by this addition, but instead slightly stimulated.

The isotope transfer observed in these experiments may possibly be connected with an amino acid exchange between the soluble RNA and microsomal proteins [8]. If there were such a coupling, a stimulation of the isotope transfer under the influence of added amino acids might possibly be expected. A stimulation was actually observed in a few experiments, but the effect was not considered significant. As a matter of fact, the critical amounts of amino acids needed for an exchange may conceivably be released from the microsomal particles in the course of the reaction.

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INCORPORATION DE THYMIDINE TRITIÉE DANS L’OVAIRE DES GRYLLIDES (ORTH.)

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Reçu le 30 juin, 1958

’EXISTENCE de précurseurs de l’acide désoxyribonucléique (ADN) dans les œufs
erges d’espèces très différentes paraît aujourd’hui admise [1]. On ne possède ce-
ndant que des données fragmentaires sur leur constitution [6], leur participation
u développement [10, 11], leur origine et leur localisation. Nous avons précédemment
ontré [2] que l’extraction des acides nucléiques d’un broyat d’œufs vierges de *Gryllus*
maculatus donne, après élimination des fractions acidosoluble et acide ribonucléique,
n résidu protéique contenant environ 0.001 μg de thymine par oeuf. La teneur de
vésicule germinative n’excédant vraisemblablement pas 12^{10-7} μg de thymine,
résultat s’ajoute aux données du même ordre citées par J. Brachet [1]. Nous avons
sayé de préciser ces données en étudiant par autoradiographie [3] l’incorporation
thymidine tritiée dans l’ovaire des Gryllides.

Matériel et Méthodes.—Des femelles adultes de *G. bimaculatus* ont été injectées
ec une solution contenant 18 μc/ml de thymidine tritiée[3], à raison de 1.5 μc par
jection. Fixation, mise en place de l’émulsion, développement et coloration suivant
technique de Ficq [3]. A titre de contrôle, on a utilisé l’acide perchlorique froid [8]
la désoxyribonucléase, dans les conditions habituelles.

Résultats.—Signalons, en premier lieu, que le traitement des préparations par l’acide
rchlorique dilué n’affecte pas sensiblement leur radioactivité. Toutes les prépara-
ons ont donc été préalablement traitées de cette façon. Notons ensuite que la radio-
tivité qui résiste à l’extraction acide est totalement supprimée par la désoxyri-
nucléase, tandis que les lames témoin ne subissent que de faibles modifications.
est donc logique d’attribuer la radioactivité des zones marquées de la préparation
l’incorporation de la thymidine dans l’ADN [4]. La répartition des zones permet
faire les observations qui suivent.

A aucun stade, même après une longue période d’incubation, on n’observe la moin-
e activité dans le noyau de l’oocyte. Ce fait paraît devoir exclure la vésicule ger-
inative comme siège de la synthèse du composé contenant la thymine précédem-
ent dosée dans l’œuf mûr.

Les noyaux des cellules folliculeuses sont diversément marqués: ceux des cellules
lliculeuses qui entourent les jeunes ovocytes en période d’accroissement cyto-
asmique ne se marquent qu’à la mitose. Pendant la vitellogénèse, et tandis que

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[1] Adresse permanente: Faculté des Sciences. 12, rue Cuvier, Paris 5, France.

[2] J’exprime ma vive gratitude à la commission des Echanges culturels Franco-Belge du Minis-
e de l’Instruction publique de Belgique et aux Commissions du C.N.R.S., qui par l’octroi
ne bourse ont rendu possible un stage dans le service du Prof. Brachet.

[3] Tritiated thymidine. Schwarz Lab. Inc., Mt Vernon, N.Y.

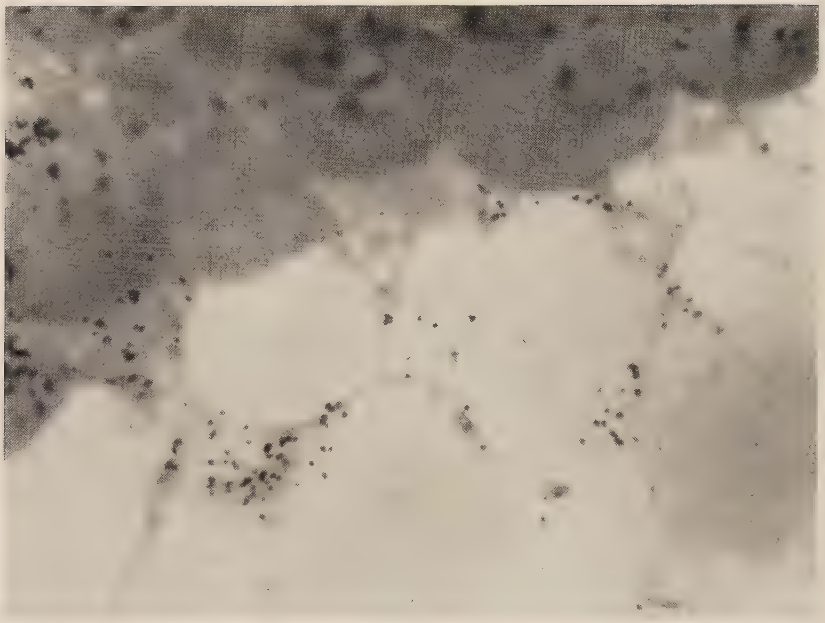

Fig. 1.—Autoradiographie d'une coupe intéressant les cellules folliculeuses et une partie d'un oocyte de *Gryllus bimaculatus*, pendant la vitellogénèse, 24 heures après injection de thymidine tritiée. La radioactivité est localisée au niveau de la trame cytoplasmique, autour des plaquettes vitellines.

ces noyaux ne subissent plus ni mitoses ni amitoses [7] mais seulement un fort accroissement de volume, ils incorporent massivement la thymidine. Cette augmentation de l'incorporation va de pair avec une forte affinité pour le vert de méthyle, une Feulgen posivité intense, des modifications de forme et d'orientation, ainsi qu'avec un accroissement considérable du volume du nucléole. Ce dernier n'est cependant jamais à l'origine de la radioactivité. Cette incorporation n'affecte pas simultanément toutes les cellules d'un même follicule, certains noyaux étant radioactifs, tandis que d'autres, de même aspect, ne sont pas marqués. Cet asynchronisme de leur fonctionnement n'est sans doute qu'un aspect de celui qui affecte l'activité sécrétrice de plages entières de cellules folliculeuses [7]. Des observations similaires ont été faites par Ficq sur les ovaires de Batraciens [5]. Si l'injection de thymidine est faite dans une femelle plus âgée, où les oocytes les plus avancés sont déjà isolés dans leur chorion, les noyaux des cellules folliculeuses qui ont participé à la sécrétion de ce dernier ne sont pas marqués.

On peut enfin observer que le cytoplasme des cellules folliculeuses ayant atteint leur taille maximum, ainsi que celui de l'oocyte, qui est réduit à l'état d'un fin réseau basophile enserrant les plaquettes de vitelles protéique, présentent un certain marquage. Fig. 1 montre l'existence de grains d'argent réduit, immédiatement au-dessus et le long de cette trame basophile, et au-dessus des prolongements cytoplasmiques s cellelules folliculeuses qui, à ce stade, sont étroitement en contact avec le cyto- sme de l'oocyte. Rien de tel n'est observable après action de la désoxyribonucléase. est tentant d'admettre qu'il s'agit là d'images concrétisant la migration, depuis noyau des cellules folliculeuses jusqu'au cytoplasme de l'oocyte, d'une substance acidosoluble et contenant de la thymidine. Cette interprétation aurait l'avantage rendre compte des résultats des dosages de thymine.

Cette conclusion doit cependant être accompagnée de certaines réserves: les ects nets ne se rencontrent que dans les zones où sont rassemblés un certain nombre cellules dont le noyau est fortement marqué. L'absence de radioactivité dans le oplasme des cellules dont le noyau n'est pas marqué paraît exclure l'hypothèse ne fixation non spécifique du traceur, mais elle évoque la possibilité d'un artefact: partie de la radioactivité du cytoplasme pourrait être due au fait qu'un site forte radioactivité, comme le noyau, tend à impressionner une surface d'émulsion siblement supérieure à la sienne propre. Mais on ne peut pas expliquer ainsi istence de grains d'argent rassemblés par petits groupes, à bonne distance du yau, et dans un plan optique qui interdit toute confusion avec les grains de fond l'émulsion. Dans l'oocyte en vitellogénèse, ce n'est qu'aux endroits où la trame oplasmique est en bon état que ces figures sont observables. Cette trame est mal servée par la fixation drastique qu'exige la sensibilité de l'émulsion photographi- , et elle est sérieusement désorganisée par la dissolution du vitellus lipidique dant l'inclusion. Notons enfin que le phénomène illustré par la figure 1 paraît ité dans le temps à la période de pleine activité sécrétoire des cellules folliculeuses.

Quoiqu'il en soit, la participation des cellules folliculeuses à l'accumulation dans ytoplasme de l'oocyte de produits dérivés de l'ADN de leur noyau et riches en mine paraît vraisemblable. Ce processus n'exclut pas l'éventuelle absorption par cyte de cellules folliculeuses entières, comme le laissent supposer d'autres observa- s. Un mécanisme comparable a été décrit chez les Hémiptères [9]. Chez les Gryl- s, on notera qu'il paraît n'intéresser que des cellules dont le noyau est incapable corporer la thymidine.

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PERMEABILITY OF RABBIT, RAT AND HAMSTER EGG MEMBRANES

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Received June 30, 1958

THE effective environment of the mammalian egg may be contributed to by any substance capable of passing through the secondary and tertiary membranes—the zona pellucida, cumulus oophorus and mucin coat. Consequently, to appreciate the nature of the effective environment of eggs under natural or artificial conditions it is necessary to have a knowledge of the permeability of the encircling membranes. Accordingly, we have attempted to assess the pore size of these membranes in rabbit, rat and hamster eggs, and the results are set out in this brief communication.

The only previous study of this kind appears to be that of Dean and Moore [1], who worked with the eggs of the sea urchin *Dendraster*. They determined the permeability of the fertilization membrane by placing the eggs in hypertonic solutions of substances of high molecular weight and noting whether the membrane returned to its former shape after initial collapse. The *Dendraster* fertilization membrane was found to admit solutes of a molecular weight of 4000 or less but not of 10,000 or more.

As the relevant mammalian membranes are virtually incapable of collapse, Dean and Moore's method was not suitable for our purposes. Dependence was therefore placed upon the use of substances that could be detected in some way after their passage through the membranes, and the most appropriate were found to be digitonin (B.D.H.), the dyes toluidine blue (Gurr) and alcian blue (Gurr),[1] and heparin (B.D.H.).

Methods.—The tests were conducted in 0.9 per cent NaCl solution and the test-substances were dissolved in the same medium. Digitonin and alcian blue were used as saturated solutions, toluidine blue as a 0.1 per cent solution, and heparin as a 1 per cent solution.

For the tests with digitonin, toluidine blue and alcian blue, the cumulus masses or eggs were placed on a slide and covered with a coverslip with vaselined edges. The coverslip was lowered until the eggs were slightly compressed, and the test solution was then drawn under the coverslip. The arrival of digitonin at the vitelline surface was shown by distinct alterations in the cytoplasm, notably the development of protrusions and the disintegration of the cortex. The passage of the dyes through the egg membranes was very easily followed under the microscope at a magnification of 100 ×.

To determine whether membranes were permeable to heparin, two procedures were employed: the eggs were allowed to stand in about 1 ml of the heparin solution overnight (16–20 hr), washed, and then treated with toluidine blue, or else they were first treated with the toluidine blue and then allowed to stand overnight in the heparin solution. It was assumed that, by either procedure, the entry of heparin would be marked by the development of a strongly metachromatic colour reaction in the thickness of the membrane or in the perivitelline space.

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[1] Sample kindly donated by E. Gurr & Co., London.

Observations.—Rabbit eggs. Cumulus masses were recovered from a rabbit 6–7 hr ter artificially induced ovulation and treated with alcian blue. Penetration of the e occurred very slowly, advancing 50–100 μ overnight. Rabbits were killed 1, 2 d 3 days after insemination, and 6, 7 and 5 fertilized eggs, respectively, were recov-ed and tested. The 1-, 2- and 3-day eggs had mucin coats with average thicknesses about 23 μ, 68 μ and 159 μ, respectively; the average thickness of the zona pellucida as about 15 μ. Digitonin readily penetrated to the vitellus in all eggs treated with is reagent, but the rate of passage could not be measured accurately because the toplasmic reaction developed gradually. Alcian blue reached the perivitelline space most immediately in the one-day eggs, in about 10 sec. in the two-day eggs and in –60 sec. in the three-day eggs. There was no evidence of penetration of the heparin lution.

Rat and hamster eggs.—Cumulus masses recovered at an estimated time of 5–10 after ovulation, were tested with the alcian-blue solution. Penetration was slow: ter 20–24 hr the dye had advanced a distance of 50–100 μ through the matrix of e cumulus. On the other hand, toluidine blue permeated the whole cumulus within few minutes.

Toluidine blue, digitonin and alcian blue were also observed to pass through the na pellucida. This was seen in a total of 9 unfertilized rat eggs, and 5 unfertilized mster eggs recovered within 12 hr after ovulation and denuded with hyaluronidase, d in 8 fertilized 2-cell rat eggs and 6 fertilized 2-cell hamster eggs. Passage of the agents was so rapid that it could not be accurately timed nor could any difference detected between fertilized and unfertilized eggs. Twelve 2-cell rat eggs tested th heparin solution showed no evidence that this substance could pass through the na pellucida.

Discussion.—The results obtained are quite simple and clear cut: the cumulus phorus and zona pellucida of rat, hamster and rabbit eggs, and the mucin coat rabbit eggs were permeable to toluidine blue, digitonin and alcian blue solutions. e mucin coat of rabbit eggs and the zona pellucida of rat and rabbit eggs were parently not permeable to heparin solution. The rates of passage of the dyes sug-sted that the zona pellucida and mucin coat were more permeable than the cumulus phorus. The molecular weights of toluidine blue, digitonin, alcian blue and heparin e, respectively, about 300, 1200, 1200 and 16,000. Thus, the membranes have en demonstrated to be permeable to substances of molecular weight 1200 or less d not to 16,000. The pore size would therefore be in roughly the same range as at of the *Dendraster* fertilization membrane.

From considerations of molecular weight alone, it is concluded that the vitellus living rabbit, rat and hamster eggs throughout their tubal existence, or in culture the corresponding stages, should be directly accessible to all the known essential od constituents, including vitamins, to the great majority of pharmacologically tive compounds and toxins, and to all the natural steroid hormones. On the other nd, the membranes are evidently impermeable to most enzymes, antigens, anti-dies, protein-containing hormones, and to substances of the nature of the in-rtebrate fertilizins and antifertilizins.

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SURFACE FINISH INTERFERENCE MICROSCOPY OF BIOLOGICAL OBJECTS[1]

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Received June 30, 1958

With the surface finish interference microscope furnished with suitable dielectric objectives, as for example in the Baker instrument, it is possible to measure the height, or depth, of fixed stained or unstained biological objects to within a fraction of the wavelength of the illuminating light.

When a plane surface is viewed in this microscope the field will be seen traversed by equally spaced parallel fringes. As any linear separation between two adjacent fringes always indicates a difference in vertical direction of $\lambda/2$ (for green mercury light $= 0.273\ \mu$), the width and linear separation of the fringes will depend upon the degree of tilt in relation to the optical axis, and the fringe direction will depend upon the direction of the tilt. If the surface has flaws and defects, a deformation of the fringes will take place. The pattern of this deformation will depend upon the shape of the object and its orientation in relation to the optical axis. The extent of the fringe deformation gives an absolute measurement of the difference in vertical direction between the adjacent surface and the measured point. Thus the tail of the fixed, unstained mouse spermatozoon in Fig. 1, has a height (= thickness) at the point C of $\lambda/2 \times AC/AB = 0.273 \times 0.83 = 0.23\ \mu$. If the object is spherical with a radius $R\mu$, the fringe deformation will form a system of concentric circles, the number of which will be $R/0.273$, and their radius will be $\sqrt{R^2 - (0.273 \times n)^2}$, where n is an integer having the values 1, 2, 3, ... n. Fig. 2 shows the appearance of nuclei from films of suspensions of kidney and liver nuclei from the same mouse, isolated simultaneously using the method given by Frazer and Davidson [1]. The films of suspensions were dried at room temperature, fixed for two hours in acetic acid–ethanol (1:3), rinsed for 6 hours in running tap water and then dried at room temperature. Fig. 2A and B show liver nuclei, 2C and D kidney nuclei, and 2E a (Feulgen stained) liver nucleus seen under greater tilt. From the interference patterns it can be deduced that nuclei prepared in this way rest flatly on the slide with an upward convexity.

The height at any point of the nuclei, viewed at normal incidence, can be determined by counting the number of fringes from the periphery to the point in question. This can be done either densitometrically or visually. In both cases the accuracy will depend upon the accuracy with which fractional fringes can be determined. The distance between a dark and a light fringe is 1/2 fringe ($= 1/4\ \lambda$); if therefore the fractional fringe is determined to the nearest dark or light fringe the maximum percentage error will be $25/n$, where n is the number of dark and light fringes counted. As the occurrence of the nuclear maximum thickness being an exact multiple of $\lambda/2$ is a rare event, the occurrence of fractional fringes will be the rule. It is, however,

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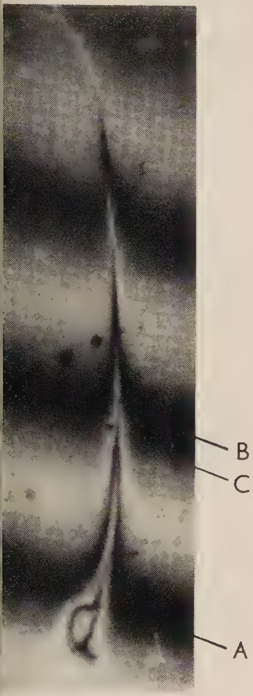
[1] This investigation was supported by a grant from the British Empire Cancer Campaign.



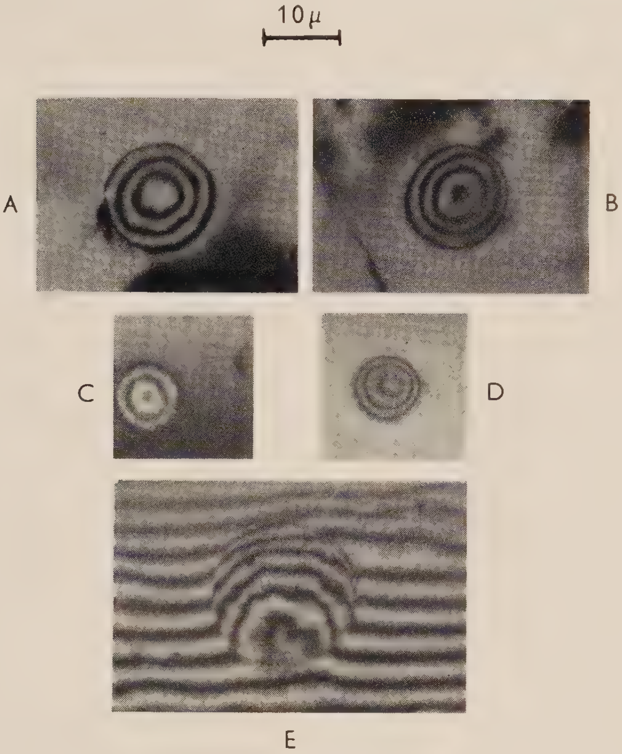


Fig. 1.

Fig. 2.

ig. 1.—Fixed, unstained mouse spermatozoon. Total magnification 1150 × .

g. 2.—*A–B*, fixed, unstained mouse liver nuclei. Polar axis parallel to the optical axis. *C–D*, xed, unstained mouse kidney nuclei. Polar axis parallel to the optical axis. *E*, fixed, Feulgen ained mouse liver nucleus. Polar axis not parallel to the optical axis.

ossible to place the nucleus in such a way on the stage that, while retaining normal cidence, a light or a dark fringe will fall on the centre of the nucleus. In this case ie only occurring fractional fringe will be at the periphery, where the curvature is eatest.

Fig. 3 is a scatter diagram of the visually determined maximum thickness (to e nearest 1/2 fringe) and diameter (to the nearest 1/4 mm) measured on enlargeents giving a total magnification of 1150 × of 36 randomly chosen kidney nuclei d 36 liver nuclei from the preparations mentioned above. As it is seen from this agram the kidney nuclei have a smaller diameter (average 7.32 μ; st.e. $= 0.1393$) ian the liver nuclei (average 12.17 μ; st.e. $= 0.3100$). As the degree of variation in ameter amongst the two types is not comparable ($F = 4.95$), a t-test for the differce between the two means is not applicable. The maximum thickness of the two pes seems to be similar as judged by the diagram. The average maximum thickness r the kidney nuclei is 2.875 fringe ($= 0.785$ μ); st.e. $= 0.0877$; and for the liver

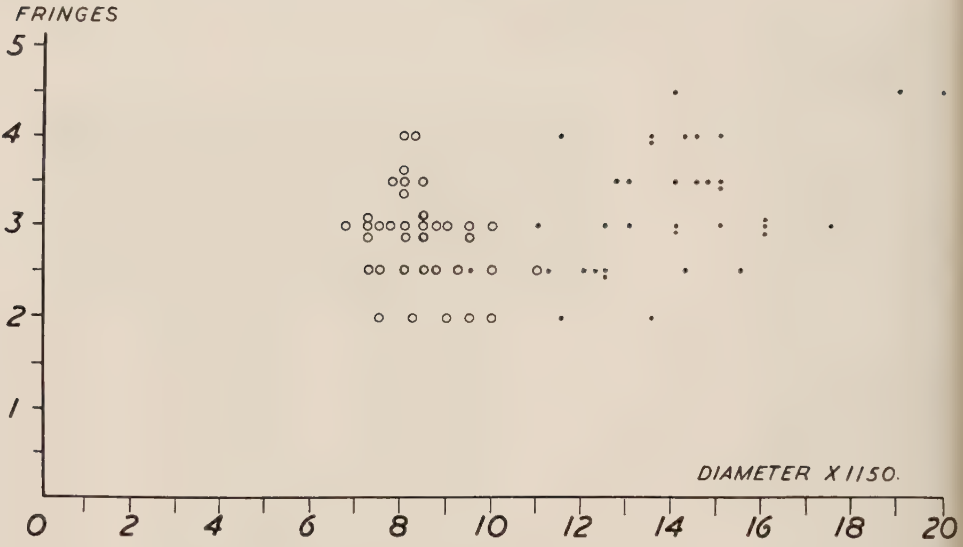


Fig. 3.—Scatter diagram of maximum nuclear thickness and nuclear diameter. *Open circles:* kidney nuclei. *Dots:* Liver nuclei. *Ordinate:* number of fringes. *Abscissa:* Nuclear diameter × 1150 (mm).

nuclei 3.222 fringe ($= 0.88\ \mu$); st.e. $= 0.1152$. The degree of variation is comparable ($F = 1.73$), and a t-test for the difference between the two means shows this to be insignificant.

The kidney nuclei have thus a greater curvature than the liver nuclei which, if preparational selection can be excluded, indicates that the two types of nuclei have either different masses or different structure.

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NOTE ON THE DISTRIBUTION OF MINERAL SALTS AND "BONE SEEKING" RADIOISOTOPES IN SPONGIOUS BONE TISSUE[1]

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Received June 30, 1958

HE knowledge of the distribution of the bone-seeking radioisotopes in the miner-ized tissues is of fundamental importance for estimating local dose rates given by dioactivity contained in the skeleton. Especially the problem of internal poisoning ith ^{90}Sr, one of the main fission products and present, among other things, in the dioactive fall out, has recently attracted a great deal of interest.

In general it can be stated that microscopic areas in bone with a comparatively w content of mineral salts show a relatively high uptake of radioisotopes, for example Ca, ^{90}Sr, ^{144}Ba, $^{32}\mathrm{PO}_4$, $^{35}\mathrm{SO}_4$ and others. These bone structures are termed "reactive one" or "reactive sites".

The content of mineral salts within microscopic bone specimens has been studied y X-ray microscopy (microradiography) both on a qualitative and quantitative asis [1]. The bone trabeculae in the spongious bone show extensive areas of low min-alization, hence these areas have a high affinity for the radioisotopes. When the azard of radiation emitted from these isotopes *in vivo* has to be evaluated, the spon-ious bone tissue can be considered critical as the radioactive bone lamellae enclose e bone marrow.

The distribution of mineral salts within such spongious bone has been studied y microradiography according to our standard procedures. The distribution of radio-ctive calcium has been studied on the same specimens, both what regards labelling *vivo* and *in vitro*. In general the *in vivo* and *in vitro* labelling give the same pattern.

In Fig. 1 is shown at a low magnification a section through a vertebral bone and the rresponding radioautogram. It is seen that the structures possessing a relatively w content of mineral salts show a considerable uptake of ^{45}Ca. This specimen was cubated in a $^{45}\mathrm{CaCl}_2$ solution for 2 hours. At higher magnification, Fig. 2, it is seen at the distribution of mineral salts within the thin bone lamellae varies and that e degree of uptake of ^{45}Ca diminishes with increasing mineralization. (In the micro-diogram the whiteness of a structure is proportional to its mineral content).

Fig. 3 shows the microradiogram and radioautogram of a section of a rib from a dog hich three days before the bone biopsy had received ^{45}Ca intraperitoneally. Also ere the uptake of isotope follows the same pattern, i.e. lowly mineralized structures ave a higher isotope uptake than well mineralized areas. In this specimen, however, e degree of mineralization is fairly homogeneous.

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[1] This investigation has in part been supported by grant D 700, National Institute of Dental esearch, National Institute of Health, Bethesda, Md., and in part by European Office of Air esearch and Development Command, Brussels, contract AF 61 (052)-15.

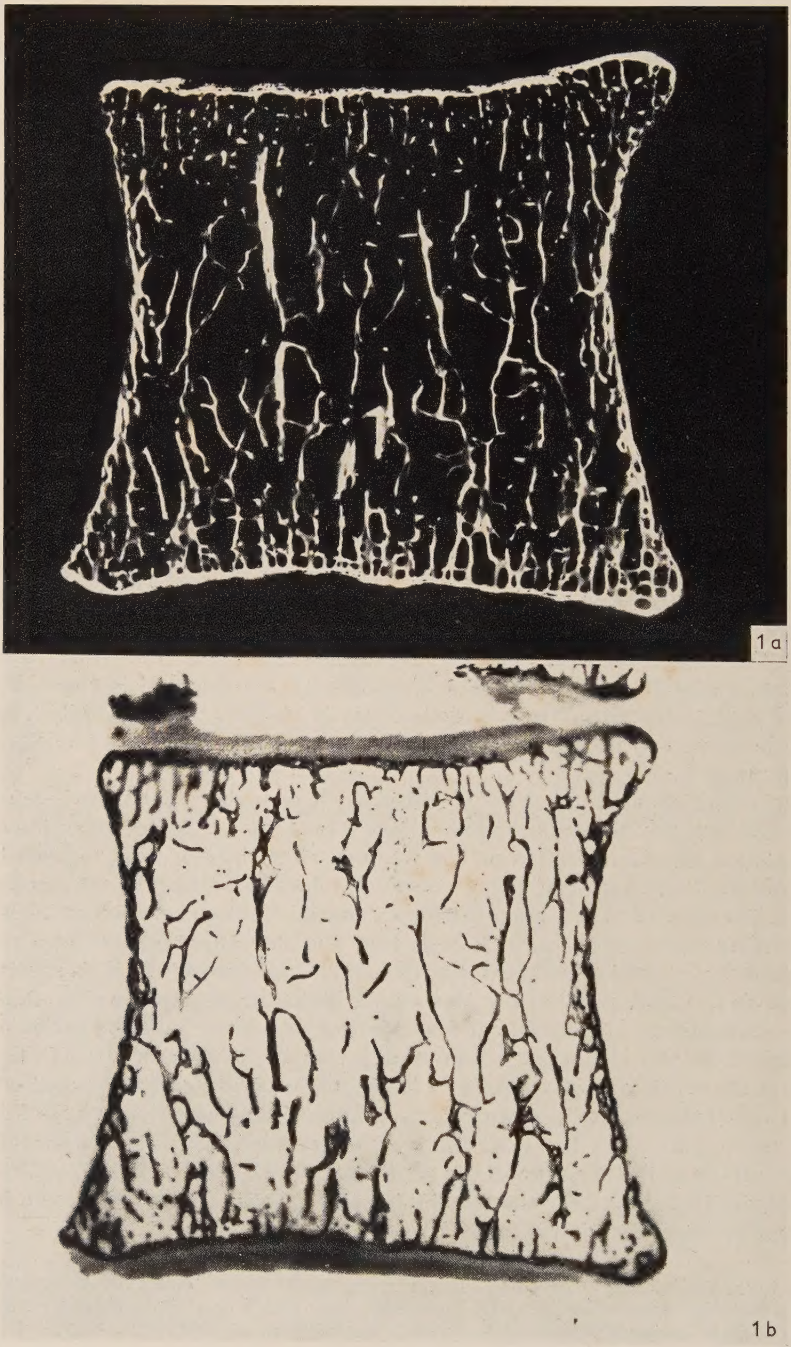
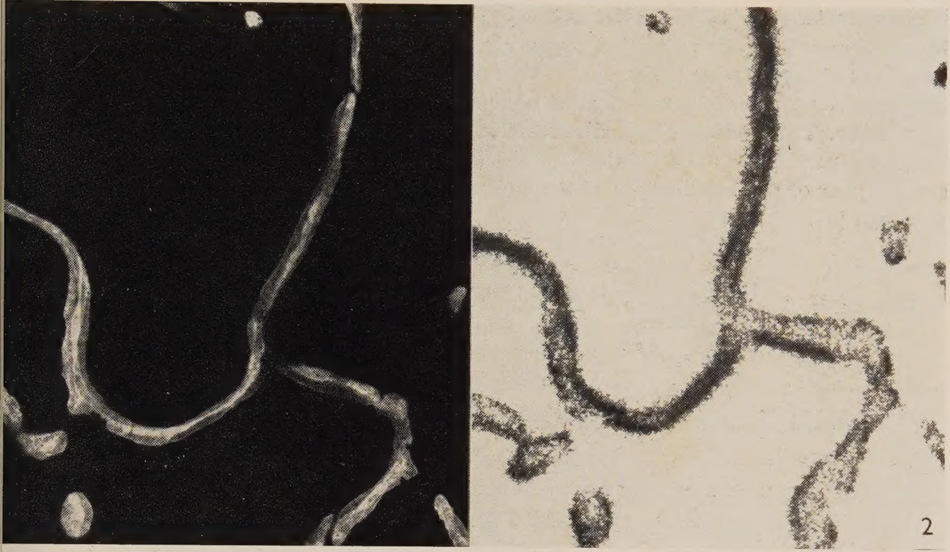
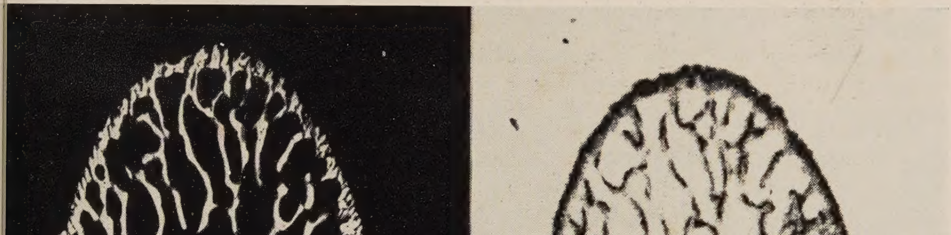


Fig. 1.—*a*, Microradiogram of a 200 μ thick section of a human vertebral bone showing the distribution of ossified tissues (white in the illustration). *b*, Radioautogram of the same section after incubation in a $^{45}CaCl_2$ solution. Mag. 4.5 ×.





ig. 2.—*Left,* Microradiogram of a few bone lamellae in spongious bone showing the varying degree mineralization within the bone structure itself. *Right,* Radioautogram of the same section ter *in vitro* labelling with ^{45}Ca. The lowly mineralized areas (greyish) have a relatively high ptake of isotope. Mag. 44 ×.

ig. 3.—*Left,* Microradiogram of a cross section of a rib from dog, given ^{45}Ca intraperitoneally efore bone biopsy. Mag. 10 ×. *Right,* Radioautogram of the same section.

Methyl green/pyronine staining was performed by the method of Dr. Brachet [3] who aided in the interpretation of the results. Following irradiation and nuclear transfers, the cells were placed in Chalkley's solution for 18–20 hours before fixation. Both the UV and the $N_i \to C_u$ cells showed shrunken nuclei with poorly developed nucleoli, together with a decrease in basophilia and a marked vacuolation of the cytoplasm. The nucleus was normal in the $N_u \to C_i$ cells, with some cytoplasmic vacuolation.

The incorporation of ^{14}C-phenylalanine (2.5 μC/ml in Chalkley's solution) over an 18–20 hour period, measured by the number of five-grain tracks or greater per unit area [9], is given relative to the controls in column 5 of Table I. These studies indicate that UV irradiation inhibits phenylalanine incorporation or exchange. The labeling occurs in both the nucleus and the cytoplasm, but mainly in the cytoplasm. The cytoplasm directly irradiated ($N_u \to C_i$) is more injured than cytoplasm of a cell exposed to an irradiated nucleus ($N_i \to C_u$). But the injury of the nucleus is transmitted to the cytoplasm as shown by decreased ^{14}C-phenylalanine incorporation or exchange in $N_i \to C_u$ cells when compared with cells subjected to nuclear transfers only ($N_u \to C_u$).

Summary.—The results obtained from survival time and methyl green/pyronine staining show that the UV injury of the cytoplasm can be reversed by replacement of an irradiated nucleus with an unirradiated nucleus, but unirradiated cytoplasm was unable to reverse the UV injury of a nucleus, unirradiated cytoplasm even being injured by the presence of an irradiated nucleus. A cell with an unirradiated nucleus in irradiated cytoplasm was inhibited in the incorporation of ^{14}C-phenylalanine more than a cell with an irradiated nucleus in unirradiated cytoplasm or a UV-treated cell without nuclear transfer.

The difference in results obtained in the ^{14}C-phenylalanine incorporation study from those expected on the basis of survival times and methyl green/pyronine staining is being investigated.

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